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İÇİNDEKİLER (Contents)

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Research Article

The Use of Various SNPs in CAST and CAPN1 Genes to Determine the Meat Tenderness in Turkish Grey Cattle [1]

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[1] *This research was presented at the 12th Turkey National Food Congress, 05-07 October 2016, Edirne, Turkey*

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Abstract

The aim of this study is to investigate the relationship between some genotypic characteristics of purebred Turkish Grey Cattle (TGC) and beef tenderness characteristics. There is a linear relationship between meat tenderness and the increasing calcium activity after slaughtering. Calpastatin (CAST) is a neutral protease inhibitor of Calpain (CAPN1) in mammalian tissues. The three polymorphic places in the CAST and the CAPN1 genes in cattle (*Uo*G-CAST, CAPN1 316 and 4751) are known as the markers of beef quality. The relationship between the tenderness traits of the *longissimus dorsi* (LD) and the 3 beef quality markers (3 SNPs) in pure TGC have been investigated and discussed. PCR-RFLP and ARMS-PCR methods were utilized to identify the genotypes. In order to determine the water holding capacity (WHC), cooking loss (CL) and the shear force (SF), samples extracted from LD were probed. The average and standard error SF of a two-year-old TCG bred in extensive conditions was 4.339±0.217 kg/cm^2 for the heifers and 4.689±0.569 kg/cm^2 for bulls. Both alleles of the *Uo*G-CAST (C/G), the CAPN1 316 (C/G) and the CAPN1 4751(C/T) polymorphisms in the samples were observed. The average SF of 3.943±0.441 kg/cm^2, 4.537±1.666 kg/cm^2, and 3.869±0.721 kg/cm^2 were used for the CAST-CC, the CAPN1 4751-CC and the CAPN1 316-GC, respectively in order to cut the muscle fibers of the genotypes that have a positive effect on tenderness. No cattle of the CAPN1 316-CC genotype was identified among the samples. The average and standart error WHC, CL and SF values for the entire sample including heifers and bulls were found as 11.693±0.761%, 26.952±0.636%, and 4.483±0.252 kg/cm^2, respectively. In conclusion, the presence of genetic variation in specific SNP markers of beef tenderness in purebred TGC can contribute to the process of raising TGC with more tender meat.

Keywords: *Beef texture, Water Holding Capacity, Cooking Loss, CAST, CAPN1, PCR-RFLP, ARMS-PCR*

Boz Irk Sığırın Et Gevrekliğini Belirlemede CAST ve CAPN1 Genlerindeki Kimi SNP'lerin Kullanımı

Özet

Bu çalışmanın amaçı saf Boz ırk sığırın (TGC) kimi genotipik özellikleri ile et gevreklik özelliği arasındaki ilişkiyi araştırmaktır. Kesim sonrası sığır etlerinde artan kalsiyum aktivitesi ile et gevrekliği arasında doğrusal bir ilişki vardır. Kalpastatin (CAST), memeli dokularında bulunan kalpain'in (CAPN1) nötr bir proteaz inhibitörüdür. Sığır CAST ve CAPN1 genlerinde üç polimorfik yer (*Uo*G-CAST, CAPN1 316 ve 4751) et kalite markörü olarak bilinir. Saf TGC'ın *longissimus dorsi* (LD) kası gevreklik özellikleri ile 3 et kalite markörünün ilişkileri araştırılıp tartışılmıştır. Genotipleri tanımlamada PCR-RFLP ve ARMS-PCR metotları kullanılmıştır. Etin su tutma kapasitesi (WHC), pişme kaybı (CL) ve tekstürünü (SF) belirlemek için LD kası örnekleri üzerinde çalışılmıştır. Extansif koşullarda yetiştirilen iki yaşındaki TGC sığırların SF ortalama ve standart hataları düveler de 4.339±0.217 kg/cm^2, erkekler de 4.689±0.569 kg/cm^2 olduğu saptanmıştır. Çalışılan et örneklerinde *Uo*G-CAST (C/G), CAPN1 316 (C/G) ve CAPN1 4751(C/T) polimorfizmlerinin her iki alleli de gözlenmiştir. Et gevrekliği üzerine olumlu etkisi olan genotiplerin, kas liflerini kesmek için ortalama CAST-CC için 3.943±0.441 kg/cm^2, CAPN1 4751-CC için 4.537±1.666 kg/cm^2 ve CAPN1 316-GC için 3.869±0.721 kg/cm^2 SF uygulanmıştır. Örneklerde CAPN1 316-CC genotipinde sığır belirlenmemiştir. Dişi ve erkek sığırlara ait tüm örneklerin dahil olduğu WHC, CL ve SF ortalama ve standart hata değerleri sırasıyla; %11.693±0.761, %26.952±0.636 ve 4.483±0.252 kg/cm^2 olarak belirlenmiştir. Sonuç olarak, safkan TGC da et gevrekliğine özgü SNP markörlerin genetik çeşitliliğin olması, daha gevrek etli TGC sığır yetiştirme sürecine katkıda bulunabilir.

Anahtar sözcükler: *Et tekstürü, Su Tutma Kapasitesi, Pişme Kaybı, CAST, CAPN1, PCR-RFLP, ARMS-PCR*

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INTRODUCTION

Meat is obtained from warm-blooded and healthy cattle, ovine and poultry by butchery. The part that is left after all the blood is drawn and the parts that are not suitable for consumption are removed is the meat on the bone. The word "meat" usually refers to the skeletal muscles, which is also called striated muscles. After the rigor mortis stage ends, the meat which has become rigid softens with the ripening when it is not sliced and becomes tender. The meat should be kept in a cool and hygienic environment to ease the effects of rigor mortis. During the ripening pH ranges between 5.2 and 6.2 in butchery animals as a result of various enzymatic activities. The meat with pH 6.4 is suspected to have bacterial decomposition. The tenderness of meat which has a huge impact on customer satisfaction is one of the most important issues in beef cattle production [1]. CAPN proteolytic system is responsible for the tenderizing process after death. CAST prevents μ- and *m*-Calpain activity and regulates postmortem proteolysis. There is a linear correlation between the calcium activity after death and the meat tenderness [2]. Many studies show that CAPN-CAST system is important to the normal development of skeletal muscles. Smith *et al*.[3] reported that the specific SNPs in CAST and CAPN1 loci are related to some genetic effects that are important to meat tenderness. Also, most reports of the same subject point out the relationship between the CAST and CAPN1 genetic polymorphism and LD and the shearing resistance of the muscle [4-6]. Instead of choosing the best meat after determining the meat quality parameters (taste, juiciness or tenderness), it would be wiser to choose the animals with the genotypes which have more qualified meat. It is possible to choose the animals with more qualified meat by determining the polymorphisms in CAST and CAPN1 gene loci of the animals without using complicated selection procedures. In *Bos Taurus* beef, there is a relationship between the SF shearing resistance and *Uo*G-CAST SNPs during the postmortem 7th, 14th and the 21st days. The cattle with *Uo*G-CAST CC genotype are likely to have more tender beef compared to the cattle with GG genotype, and the cattle with CG genotype have to medium tenderness beef [5-7]. In addition, some researchers who worked on *Bos taurus* [3,8-10] and *Bos indicus* [4,11,12] beef found a correlation between the SF values and the CAPN1 316 SNPs and 4751 SNPs on the 7th and 14th days after the slaughter ($P<0.05$). The SF applied to homozygous cattle meat with the CAPN1 316 CC genotype had 10% and 14.6% less resistance compared to the heterozygote CG genotypes and homozygous GG genotypes, respectively [13]. Beef from *Bos Taurus* cattle with the CAPN1 4751 CC genotypes are more likely to be tender compared to the cattle with other genotypes [8,14].

Some commercial genetic indicators have been developed to increase beef tenderness, such as the *Uo*G-CAST and the CAPN1 316 markers [6,14]. National Cattlemen's Beef Association in the USA has approved the efficiency of those genetic markers. The association recommends the cattlemen to increase the ratio of the CAPN1 316/4751 haplotype C/C frequency within the cattle population to have more tender beef [6].

The aim of this study is to obtain empirical data from the purebred TGC's beef (from the rib steaks) samples by comparing their WHC, CL, SF data to TGC with different genotypes which are formed by the *Uo*G-CAST (CAST/*Rsa*I), the CAPN1 316 and 4751 polymorphisms.

MATERIAL and METHODS

Animals

The samples that were extracted from 17 TGC (10 males and 7 females) from the rural cattle breeding barns in Enez District, Edirne in 2013 were used. The barns from which the samples were extracted utilized extensive breeding methods in grazing lands. In this study, female and male animals bred in Thrace and slaughtered at 24-months-old were used.

Separation and Evaluation Time of The LD

TGC brought from the rural grazing lands of Çandır village in Enez district were cut off in Keşan slaughterhouse. The LD samples of our research material were separated from TGC carcasses after 24 h of waiting in slaughterhouse.

The assessments of WHC (%), CL (%) and Warner-Bratzler SF (kg/cm^2) on the *LD* were done on the 21st day after death at Istanbul University, Faculty of Veterinary Medicine, Zootechnics Department Laboratory.

Calculation of The WHC

In order to measure the WHC "Passive Drip Loss" method [15] was used. To do this, 100 g samples were extracted from the LD muscle and weighted (weight1). The samples were placed in a polyethylene bag without touching the surface of the bag and they were weighed again after they were stored at +4ºC for 24 h (weight2). WHC was calculated with the following formula [15].

WHC (%) = [(weight1-weight2)/weight1]*100

Calculation of The CL

The meat frozen at -18ºC was thawed at +4ºC and 100 g meat samples were placed in polyethylene bags and vacuumed. The meat was cooked in water-bath for an hour until the internal temperature of the meat reached 80ºC. The cooked meat was cooled down for 12 h at +4ºC and the surface was dried before it was weighed. CL percentage was calculated with the following formula [15,16].

CL (%) = [(weight of raw steak after thawing – weight of cooked steak)/weight of raw steak after thawing] × 100.

Texture Analysis

After the calculation of CL, the cooked meat was sliced with a SF knife with a 50-kg cutting power at 20 cm/min speed to determine the shearing resistance of the muscle fibers for the texture analysis. The meat cooked at 70-72ºC were cooled down 5-6ºC and they were sliced as a section of 2.54 x 2.54 cm parallel to the muscle fibers. Six samples were extracted from each LD and they were sliced. *The highest force* (kg/cm^2) and *force* x *time* graphics obtained during the application of the SF knife for each sample were saved on the computer. The peak SF value for a muscle of an animal was calculated by averaging the values obtained from the six samples extracted from that animal [11,15].

DNA Isolation

Genomic DNA of the tissue samples taken from TGC were isolated using Fujifilm QuickGene-mini-80 device and tissue kit (Quick Gene DNA Tissue kit, Fujifilm, UK) based on the instructions given by the manufacturer. A nanodrop ($A^{260/280}$) was used to determine the amount of the DNA and the samples were stored at –20ºC in Trakya University Biotechnology and Genetics Laboratory.

Amplification of the Target DNA Samples and Identification of the Genotypes

The target DNA was amplified and The PCR-RFLP method with R*sa*I restriction enzyme was utilized to identify the genotypes of CAST gene SNP (AY008267.1:g.282C>G) according to the works of Schenkel *et al.*[5] and Curi *et al.*[11]. In order to determine the CAPN1 4751 SNP (AF 248054.2:g.6545C>T) and the CAPN1 316 SNP (AF252504. 2:g.5709C>G) genotypes, the ARMS-PCR method was used based on the work of Rincón and Medrano [17]. The primers (Sentegen Biotech, Ankara/Turkey) used in this study were given in *Table 1* and fragment sizes were amplified using the Bioneer My Genie 96 Thermal Block PCR device (Bioneer Corporation, South Korea). Amplification of DNA was run in the 2% horizontal gel electrophoresis and was used for the genotyping by "DNR BioImaging Systems Minibis Pro. Jerusalem, Israel".

Table 1. *Primary Sequences used in PCR and the product size obtained*

Marker	Primer Sequences (5′ - 3′)	bp
**UoG* CAST	[1]Fop:CTCGACTGCGTACCAATTCCGAAGTAAAGCC AAAGGAACA	523
	[2]Rop: ATTTCTCTGATGGTGGCTGCTCACT	
**CAPN1 316	[3]Fip: TTTCCTGCAGCTCCTCGGAGTGGAA**G**GG	269
	[4]Rip: GCTCCCGCATGTAAGGGTCCA**G**GG	228
	[1]Fop: GCTGTGCCCACCTACCAGCATC	446
	[2]Rop: CAGGTTGCAGATCTCCAGGCGG	
**CAPN1 4751	[3]Fip: GCATCCTCCCCTTGACTGGGGGGAAA**C**CC	158
	[4]Rip: GTCACTTGACACAGCCCTGCGCC**G**CA	231
	[1]Fop: CCTGGAGTCCTGCCGCAGCATGGTCAAC	334
	[2]Rop: AAGCTGCAGGAGCTGCCCAAAGCCAGGC	

** RFLP method, ** ARMS method, [1] **Fop:** Forward outer primer, [2] **Rop:** Reverse outer primer, [3] **Fip:** Forward inner primer, [4] **Rip:** Reverse inner primer, **bp:** Base pair*

Statistical Analysis

We genotyped TGC based on the polymorphic The *Uo*G-CAST loci in CAST gene and the CAPN1 316 and 4751 loci in CAPN1 gene. After genotyping, the samples underwent phenotypic analysis to calculate WHC%, CL% and SF (kg/cm^2). Averages (X), standard error (s_x), the genotypic and phenotypic correlations (r) were calculated using SPSS statistics program. In addition, two-tailed t-test was used to compare the results. According to the Hardy-Weinberg (HW) equilibrium, the fit test of the distribution of Allele and genotype frequencies was calculated by the Chi-square (χ^2) test. The distribution of genotypic frequencies was evaluated based on the 5% significance level of the Chi-square table.

RESULTS

Cattle with one combination of the two different patterns one of which is matched with either homozygous or heterozygote genotypes in the *Uo*G-CAST, the CAPN1 316 and 4751 loci were identified and the three unique band patterns of the cattle genotypes (except the CAPN1 316 CC genotype) were observed in the electrophoresis gel. The genotype and allele frequencies of the 3 different SNPs of the sample 17 purebred TGC were calculated in accordance with HW equilibrium, which are shown in *Table 2*.

Two genetic variances (C and G) were identified in The *Uo*G-CAST polymorphism structure of TGC samples. According to the pattern images formed on the electrophoresis gel; homozygous CC genotype, homozygous GG genotype; and heterozygotes-GC were characterized and defined to have one band with 523 bp, two bands with 257 and 266 bp and three bands with 523, 257 and 266 bp, respectively. In all of the TGC samples the frequency of the *Uo*G-CAST C allele, which is thought to have a positive effect on beef yield, is 0.559 and the distribution between C/G alleles is in HW equilibrium ($P>0.05$). The *Uo*G-CAST genotypic frequencies of the male samples were significant ($P<0.05$) and in male samples were found to have a higher frequency of C allele (0.714). The genotypic frequencies of CC, CG and GG in TGC were observed as 0.312, 0.493 and 0.195, respectively *(Table 2)*. TGC is in HW equilibrium for the *Uo*G-CAST SNP ($P>0.05$).

In the TGC, the CAPN1 316 polymorphism genotypes were defined based on the patterns they form on the electrophoresis gel with the use of ARMS-PCR method. The ones forming 3 bands with 228, 269 and 446 bp were defined as Heterozygote CG genotype while the ones

Table 2. *The UoG-CAST (Calpastatin), the CAPN1 (μ-Calpain) 316 and 4751 allele and genotype frequencies of TGC samples*

Cattle Race	Animal Number	*Uo*G-CAST Allele Frequency		*Uo*G-CAST Genotype Frequency (n)			*Chi-square*
Turkish Grey Cattle	17	C	G	CC	CG	GG	0.529
		0.559	0.441	0.312 (5)	0.493 (9)	0.195 (3)	
		CAPN1 316 Allele frequency*		CAPN1 316 Genotype frequency* (n)			
		C	G	CC	CG	GG	36.41*
		0.059	0.941	0.003 (0)	0.111 (2)	0.886 (15)	
		CAPN1 4751 Allele frequency		CAPN1 4751 Genotype frequency (n)			
		C	T	CC	CT	TT	1.582
		0.412	0.588	0.171 (2)	0.485 (10)	0.346 (5)	

** The population is not in HW equilibrium (P<0.05)*

Table 3. *The comparison of the results related to WHC %, CL % and Meat Texture (SF kg/cm²) of the LD samples based on Gender, the UoG-CAST, the CAPN1 4751 and 316 genotypes in all TGC*

Phenotypic Features	All Group	Sex Group		UoG-CAST Genotype		
		Female	Male	CC	GC	GG
SF(kg/cm²) (X±s_x)	4.483±0.252	4.339±0.217	4.689±0.569	3.943±0.441	4.488±0.298	5.55±0.633
CL % (X±s_x)	26.952±0.636	27.505±0.929	26.161±0.866	26.688±1.108	27.584±1.015	25.793±1.560
WHC % (X±s_x)	11.693±0.761	12.687±1.082	10.272±0.947	10.188±0.969	11.667±1.293	14.772±0.740
Phenotypic Features	**All Group**	**CAPN1 4751 Genotype**			**CAPN1 316 Genotype**	
		CC	CT	TT	GG	GC
SF(kg/cm²) (X±s_x)	4.483±0.252	4.221±0.873	4.537±1.666	4.986±1.303	4.565±1.102	3.869±0.721
CL % (X±s_x)	26.952±0.636	24.385±2.123	27.688±2.730	26.506±2.530	27.068±2.866	26.077±0.270
WHC % (X±s_x)	11.693±0.761	12.406±4.843	11.850±3.568	11.093±2.554	11.644±3.251	12.060±3.251

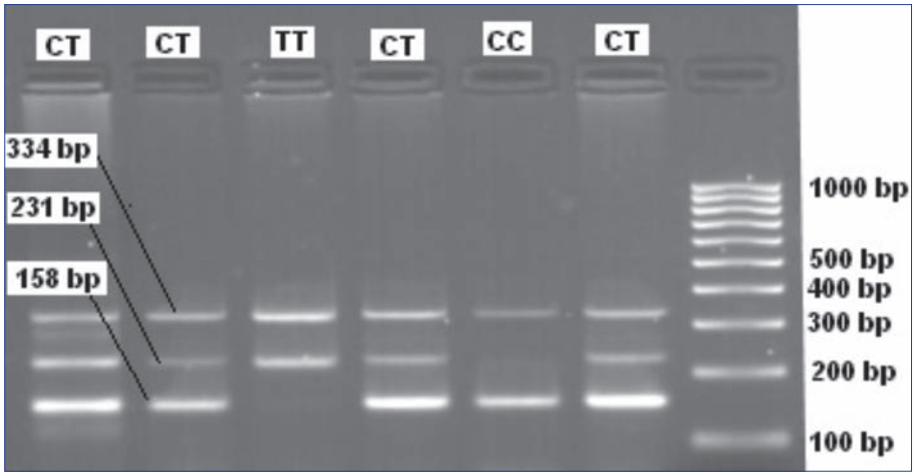

Fig 1. The agarose gel electrophoresis images of TGC with the CAPN1 4751 SNP genotypes using ARMS-PCR method

forming 2 bands with 269 and 446 bp long were defined as Homozygous GG genotype. However, there were no TGC carrying 2 bands with 228 and 446 bp that define the homozygous CC genotype in our sample. The CAPN1 316 C/G allele frequencies distribution of the samples is significant (P<0.05). There were no cattle with CC genotype in our TGC sample group and the C allele frequency which has a positive effect on the tenderness of the beef was quite low (0.059). TGC is not in HW equilibrium for the CAPN1 316 SNP (*P*<0.05) *(Table 2)*.

In the TGC, the CAPN1 4751 SNP with Homozygous CC genotype, Heterozygote CT genotype and Homozygous TT genotype were observed to form two band patterns with 334 and 158 bp, three band patterns with 334, 231 and 158 bp and two band patterns 334 and 231 bp size, respectively in the electrophoresis gel with the use of ARMS-PCR method *(Fig. 1)*. The distribution of the CAPN1 4751 C/T allele frequencies in TGC samples are in HW equilibrium. Similarly, the distribution of the CAPN1 4751 genotypic frequency was found to be in HW equilibrium (*P*>0.05).

Table 4. *The correlations derived for meat quality features and SNPs*

Traits	UoG CAST	CAPN1 4751	CAPN1 316	WHC (%)	CL (%)	SF (kg/cm²)
*Uo*G-CAST						
CAPN1 4751	0.155					
CAPN1 316	-0.052	0.400				
WHC (%)	-0.126	0.135	0.043			
CL (%)	0.015	-0.106	-0.122	0.263		
SF (kg/cm²)	-0.227	-0.221	-0.216	0.148	-0.199	
Gender	0.262	**0.536***	0.306	0.379	0.252	-0.166

** In bold, significant values (except diagonal) at the level of significance P<0.05 (two-tailed test)*

The ratio of heterozygous TGC was observed in higher frequency than other genotypes with the CAPN1 4751 *(Table 2)*.

WHC, CL and SF values for the LD samples of TGC genotypes with the UoG-CAST, the CAPN1 4751 and 316 are presented in *Table 3*. According to the results of the study, TGC with the *Uo*G-CAST CC (3.943±0.4414 kg/cm²) genotype have more tender meat compared to TGC CG and GG genotypes. Also the statistical relationship was found that between the SF value after death and the *Uo*G-CAST CC SNP genotype ($P<0.05$) of the LD muscle. The SF values of TGC with the CAPN1 316 GC genotype were found 3.869±0.7214 kg/cm². TGC with the CAPN1 316 GC genotypes were determined to have more tender meat than the GG genotypes (4.565±1.1025 kg/cm²). The difference between each other measurement was found statistically insignificant ($P>0.05$). According to the SF values of TGC with the CAPN1 4751 CC (4.221±0.8734 kg/cm²) in our sample, TGC with CC genotype were found to yield more tender meat compared to the cattle with CT and TT genotypes although the difference was not statistically significant ($P>0.374$).

The marker combinations associated with meat tenderness in TGC samples were compared. TGC with the toughest meat in our sample was the male cattle with GG/TT/GG genotype where the *Uo*G-CAST "GG", the CAPN1 4751 "TT" and the CAPN1 316 "GG" combinations merge with 6.557 kg/cm² SF value. On the other hand, the male cattle with CC/CT/GG genotype seemed to have the tenderest meat with 2.329 kg/cm² SF. The haplotype C/C/C frequency, which is positive for meat tenderness, in our TGC sample was found 0.014. The triple genotypic combinations affecting the meat tenderness in a positive way were not evaluated statistically in our sample due to their inadequate number in our sample. Except for the linear correlation between TGC with the CAPN1 4751 genotype and gender ($P<0.05$), the analysis results for the meat quality and SNP correlations did not yield a significant difference between SF, CL, WHC, gender, the *Uo*G-CAST, the CAPN1 316 and 4751 poly-morphisms *(Table 4)*.

DISCUSSION

The frequency of C allele in the *Uo*G-CAST locus is reported to be 0.74-0.79 in *Bos taurus* genetic groups [6], 0.69 [11], 0.72 [18] and 0.75 [19] in *Bos taurus* crossbreeds, 0.64 [20] in Aberdeen Angus cattle, 0.61 in Pirenaica cattle and 0.70 in Parda de Montana [21], 0.623 on average in Aberdeen Angus, Limousine, Charolais and Simmental breeds [5], 0.623 in Nellore-*Bos indicus* [11]. The C allele frequency in our sample which is made up of purebred TGC is 0.559, which is also lower than the frequencies reported by previous researchers [20,21]. For the *Uo*G-CAST SNP genotypes Gill *et al.*[22] found the CC, CG and GG genotypic frequencies in Aberdeen Angus cattle as 0.41, 0.47 and 0.12, respectively. On the other hand, Schenkel *et al.*[5] found them as 0.430, 0.398 and 0.172, respectively for *Bos taurus* while Curi *et al.*[11] found 0.377, 0.491 and 0.132, respectively for purebred *Bos indicus* Nellores. In our study, the ratio of the purebred TGC with CC genotype which expected to beef with more tenderness is lower than previous researches [20,21], except Kök *et al.*[23]. The CAPN1 316 C allele is associated with meat tenderness. TGC with the CAPN1 316 C allele frequency in our study is close to *Bos taurus* x *Bos indicus* crossbreeds [11] and similar to that of Pirenaica and Parda de Montana breeds. The frequency ratio of the TGC with the CAPN1 316 CC genotype is quite low. Except for Aberdeen Angus [22] and Hanwoo cattle [24], *Bos taurus* with CC genoype have not been observed in previous studies [11,25,26]. The CAPN1 4751 C allele frequency (0.412) that is considered to have a favourable impact on the beef tenderness seems to be higher than the other breeds except for Aberdeen Angus (0.65) [22] and Hanwoo cattle (0.80) [24]. TGC with the CAPN1 4751 CC genotype frequency (0.171) were found to be higher compared to Nellore (0.00) and Canchim (0.07) [4], Simmental and their crossbreeds (0.05) [26]. The ratio of TGC with CC genotype, which is favourable for meat tenderness, is lower than Brahman crossbreeds (0.53) [26], Aberdeen Angus (0.41) [22] and Hanwoo cattle (0.64) [24] populations.

Lee *et al.*[27] who worked on Hanwoo cattle found the average SF as 5.803±0.275 kg cm², for the heifers as 6.598±0.265 kg/cm², for the bulls as 7.200±0.275 kg/cm² and 3.400±0.061 kg/cm² for calves. Soysal [28] who worked on the texture of beef stated that gender and muscle factor do not seem to be statistically significant ($P>0.05$). When the result of the study is compared to Yüksel *et al.*[29] who worked on East Anatolian Red (EAR) breeds and found (6.84-9.46±0.49 kg/cm²) and to Özlütürk *et al.*[30] who found (5.76±0.58 kg/cm²), the meat obtained from TGC can be said to have higher levels of tenderness than EAR. Soysal's [28] applied more force to the meat of the gender groups, (4.74±0.461 kg/cm²) and female (4.94±0.393 kg/cm²), compared to the ones in our study; however he applied less force compared to the cattle with GG genotype. The difference between the Sosyal's [28] TGC sample and ours may be the genotypic differences or the nutrition of the animals. The SF values (9.95–9.97±0.23 kg/cm²) that Preziuso *et al.*[31] obtained from

Limousine cattle and Limousine crossbreeds were two times higher than the values Soysal's [28] (4.85±0.294 kg/cm^2) and the authors (4.483±0.252 kg/cm^2) of this study obtained from TGC beef samples. So, our and Sosyal's [28] TGC samples of meat can be said to have higher tenderness compared to EAR and Limousine beef according to the results of this study. Also, according to Pirenaica and Panda de Montana breeds (bulls 5.1±1.23 and 6.5±1.80 kg/cm^2, respectively) LD muscle SF values, TGC breeds are estimated to have more tender meat than Pirenaica and Parda de Montana bulls although they have tougher meat. When the average SF of TGC samples (4.483±0.252 kg/cm^2) in our study is compared to that of 15 European cattle breeds (5.49±1.25 kg/cm^2) [32] and EAR, TGC seem to have more tender meat. The shearing force applied to the LD muscle of Hanwoo cattle is [27] (-1.32 kg/cm^2) less than the average SF value for TGC.

Previous researchers reported that less force was applied to the LD muscle of the cattle with the *Uo*G-CAST CC genotype than the cattle with CG and GG genotypes and those cattle with CC genotype have more tender meat [5,6,11,22]. SF was applied to LD of Aberdeen Angus, Limousine, Charolais, Simmental and their crossbreeds on the 21st day after death as avarage 4.53±0.12 kg/cm^2 for with the *Uo*G-CAST CC and 4.91±0.15 kg/cm^2 for with GG genotype, and the difference was statistically significant (P<0.05) [5]. The SF average values of *Bos indicus* (Nellore) and *Bos taurus* x *Bos indicus* crossbreeds with the *Uo*G-CAST CC and CG genotypes were 3.47±0.007 kg/cm^2 and 3.63±0.07 kg/cm^2, respectively, and the difference was significant (P<0.05) [11]. Compared to the average SF of TGC (3.943±0.441 kg/cm^2) with the *Uo*G-CAST CC genotype, the Aberdeen Angus, Limousine, Charolais, Simmental and the other crossbreeds with the same genotype that Schenkel *et al.*[5] worked on have less tender meat than TGC; however TGC with the *Uo*G-CAST GG genotype are estimated to yield tougher meat than the other cattle with the same genotype. Also, Nellore breeds with the *Uo*G-CAST CC and CG genotypes [11] are estimated to have more tender meat than TGC with the *Uo*G-CAST GG genotype.

The SF average of Piedmontese, Simmental, Angus, Hereford, Irish, Charolais and their crossbreeds with the CAPN1 316 CC genotype found lower than the other two genotypes (GC and GG) [8,9,13,33]. Brahman breeds have a similar structure also [3,22]. Chung *et al.*[24] found the SF averages with the CAPN1 316 genotypes as CC 4.868±0.44 kg/cm^2, CG 5.180±0.22 kg/cm^2 and GG 5.437±0.27 kg/cm^2 in Hanwoo cattle, which suggests no relationship between the results and the genotypes (P=0.530). TGC breeds also have a similar structure. TGC with the CAPN1 316 GC genotype were found to have more tender meat than TGC with GG genotype but the difference (-0.696) was not statistically significant (P>0.05). We can assume that TGC with the CAPN1 316 GC genotype (3.869±0.721 kg/cm^2) are more likely to have tougher meat than the Piedmontese, Angus and Brahman breeds.

Bos taurus and *Bos taurus* x *Bos indicus* crossbreeds with the CAPN1 4751 CC genotype seem to have more tender meat compared to the other genotypes, and the difference is significant (P<0.005) [4,7,14,16]. Hanwoo cattle with the CAPN1 4751 CC, CT and TT genotypes seem to have the highest (4.946±0.19 kg/cm^2), medium (5.700±0.28 kg/cm^2) and the lowest (5.974±0.72 kg/cm^2) level of meat tenderness, respectively [24]. We can say that TGC with the CAPN1 4751 CT genotype (4.537±1.666 kg/cm^2) are more likely to have less tender meat than the Nellore breeds and *Bos taurus* crossbreeds [4], Brahman calves with the CAPN1 4751 CT [3], and also Piedmontese and Aberdeen Angus with the CAPN1 316 GG [9]. On the other hand, Gill *et al.*[20] claim these the CAPN1 4751 and the *Uo*G-CAST SNPs do not seem to affect meat tenderness in Aberdeen Angus cattle.

Ciobanu *et al.*[34] who worked on pigs and Reardon *et al.*[19] who worked on cattle could not find a correlation between the polymorphisms in CAST gene and WHC. Li *et al.*[10] who worked on the meat of Angus, Charolais, Hereford, Limousine, and Simmental calves in Sweden also could not find a meaningful relationship between the WHC properties; and some of the SNPs genotypes of CAST, CAPN1, LEP, DGAT1 and SCD1 gene. Similarly, we could not find a significant relationship between the genotypic properties of the CAPN1 or CAST SNPs and WHC or CL values of TGC.

In our research was found that the male cattle with CC/CT/GG genotype seemed to have the most tenderness meat with 2.329 kg/cm^2 SF. The previous research carry outed on Hereford, Brahman, Brangus, Red Angus cattle and Charolais X Angus crossbreeds showed that the cattle with GG/TT/GG genotype among the *Uo*G-CAST, the CAPN1 4751 and 316 triple SNPs combination had the toughest meat while the cattle with CC/CC/CC genotype tend to have the most tenderness meat [6,18]. Our findings show that TGC with GG/TT/GG genotypes have the toughest meat and this finding supports the previous studies. TGC carry SNPs that are related to meat tenderness in their CAPN1 and CAST genes, which are polymorphic. Except for the CAPN1 316 CC genotype, all CAST and CAPN1 SNP genotypes, which are associated with meat tenderness, were observed in TGC samples. Based on the SF averages which measure the resistance of the red muscle fibers, we can conclude that TGC yield more tender meat compared to East Anatolian Red, Jersey, Limousine, Pirenaica and Parda de Montana breeds. The presence of genetic variation in specific markers in TGC contributes to the process to yield more tender beef. The purebred TGC with the haplotype which includes heterozygous or double homozygous positive genes in CAST and CAPN1 genes should be kept for breeding. The effects of monadic

genes of TGC which are independent of the potential interactions with the environment on the meat tenderness, which has of economic importance and the productivity, can be designated through crossbreeding. TGC with (C/C/C) haplotype or (CC/CC/CC) genotype carrying the positive genes can be kept for breeding with the use of DNA tests to evaluate the cattle based on the *Uo*G-CAST, the CAPN1 316 and 4751 markers. In order to make TGC breeding a sustainable business, it is necessary to have cattle with more tender beef and to increase the consumption of it. Thus, there is a need to cattle which yield beef of good quality and whose genetic potential is defined. By focusing on more genes and the specific genetic indicators in these genes that are related to the quality of meat, it is possible to improve the precision in selection processes.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Intrauterine Growth Retardation Enhances Intestinal Autophagy and Proliferation in Rat Pups Responding to Colostrum

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Abstract

This study aimed to investigate responsible mechanisms for rapid intestinal catch-up growth in intrauterine growth restriction (IUGR) pups via analysis of autophagy, apoptosis and proliferation in a rat model. Twenty primiparous dams were assigned into two groups as 1) dams with feed ad libitum (Adlib) and 2) dams with 50% feed restriction from gestational day 10 to 21 to achieve normal birth weight (NBW) and IUGR pups, respectively. Litter size and pup weight were recorded at parturition and 8 pups were kept in each litter to have sufficient colostrum for 24 h. Subsequently, 2 pups from each litter were decapitated. Results indicated that feed restriction dams had similar litter size with rats in Adlib group although produced IUGR pups. Histological analysis indicated that IUGR rats had decreased villus height and surface area in jejunum. There was an accumulation of autophagosomes in jejunal mucosa of IUGR pups, however, the mitochondria and microvilli were unaffected. mRNA expressions of *WIPI1*, *MAP1LC3B, Atg13, ULK1* and *Beclin1* were increased, and *mTOR* expression was decreased in jejunum of IUGR, which also had lower *Bcl-2* mRNA expression, increased *caspase* 9 and relative increased *ki67* mRNA expression. Results suggested that after feeding colostrum, IUGR pups had impaired jejunum with unaffected mitochondrial histology. Enhanced intestinal autophagy under low-stress conditions might improve intestinal proliferation, which may be contributed to the rapid intestinal catch-up growth.

Keywords: *Autophagy, Colostrum, Intrauterine growth restriction, Intestinal proliferation, Apoptosis*

Kolostruma Cevap Veren Rat Yavrularında İntrauterin Büyüme Geriliği İntestinal Otofaji ve Proliferayonu Artırır

Özet

Bu çalışmanın amacı, rat modeli üzerinde otofaji, apoptozis ve proliferasyonu incelemek suretiyle intrauterin büyümesi kısıtlanan (IUGR) yavrularda hızlı intestinal büyümeden sorumlu mekanizmaları araştırmaktır. Yirmi adet bir doğum yapmış anneler iki gruba ayrıldı; 1) ad libitum beslenen (Adlib) anneler ve 2) Gestasyonun 10 ile 21. günü arasında yemi %50 kısıtlanan anneler. Böylece normal doğum ağırlığı (NBW) olan yavrular ve IUGR yavrular elde edildi. Doğumda yavru sayısı ve ağırlıkları kaydedildi ve her batında 8 yavru 24 saat süresince yeterli kolostrum alması için annesi ile birlikte tutuldu. Sonrasında, her batından 2 yavruya dekapitasyon uygulandı. Elde edilen sonuçlar yemi kısıtlanan annelerin yavru sayıları ile Adlib grubun yavru sayılarının benzer olduğunu ancak IUGR yavrular ürettiğini gösterdi. Histolojik incelemede IUGR ratların jejunumda azalmış villus yüksekliğine ve yüzey alanına sahip olduğu belirlendi. IUGR yavruların jejunum mukozasında otofagozomların oluştuğu ancak mitokondri ve mikrovillusların etkilenmediği gözlemlendi. IUGR yavruların jejunumlarında *WIPI1*, *MAP1LC3B, Atg13, ULK1* ve *Beclin1* mRNA ekspresyonlarının arttığı ve *mTOR* ekspresyonunun azaldığı belirlendi. Bu hayvanlarda daha düşük *Bcl-2* mRNA ekspresyonu, artmış *caspase* 9 ve orantısal olarak artmış *ki67* mRNA ekspresyonu tespit edildi. Elde edilen sonuçlar kolostrum ile besleme sonrası IUGR yavrularda histolojik olarak mitokondrilerde bir değişim olmaksızın jejunumda hasarın oluştuğunu gösterdi. Düşük stres altında gelişmiş intestinal otofaji, intestinal proliferasyonu iyileştirebilir ve bu durum hızlı intestinal büyümeye katkı sağlayabilir.

Anahtar sözcükler: *Anahtar sözcükler: Otofaji, Kolostrum, İntrauterin büyüme kısıtlaması, İntestinal proliferasyon, Apoptozis*

INTRODUCTION

Intrauterine growth restriction (IUGR) refers to the birth weight <10th percentile in a given population, which is a common and severe problem in both humans and livestock [1,2]. In America, IUGR affects more than 8% of newborns and is intimately related to metabolic disorder in adults, such as increased risk of obesity and hypertension [3,4].

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In swine industry, between 15 and 20% of neonatal piglets are affected by the growth restriction [1]. Maternal nutrition is a critical factor, which plays important roles in fetal development since both the composition of nutrients and biologically active substances through maternal placenta could affect fetal growth and are closely associated with IUGR occurrence, such as glucose and amino acids [1,5]. Therefore, the maternal feed restriction has commonly been used to construct IUGR animal models. For example, Alexandre-Gouabau et al.[6] used maternal protein restriction from the day of conception to obtain IUGR rat offspring and investigate their metabolomic responses. Gupta et al.[7] reported that maternal magnesium deficiency could alter maternal metabolism and leads fetal growth restriction.

IUGR is responsible for the relatively high rates of morbidity and mortality in neonates or fetuses, which may lead to various organ dysfunctions, and affect immune and metabolic systems [4,8]. The small intestine plays critical roles in immunity, nutrient digestion and absorption. Previous studies have documented that IUGR inhibits small intestinal development, impairs the intestinal integrity, and changes bacterial colonization [9-11]. Similarly, D'Inca et al.[12] found IUGR altered the intestinal structure of piglets with a longer and thinner small intestine and reduced villus size, while the preterm IUGR piglets provided with sufficient colostrum showed rapid intestinal catch-up growth during post-parturition period. However, the underlying mechanism of intestinal catch-up growth is still largely unclear.

Autophagy is an important process involved in cytoplasmic component degradation by the lysosome, such as damaged organelles and long-lived proteins [13]. Recently, emerging evidences have verified that autophagy is responsible for multiple biological functions, such as proliferation and apoptosis [14,15]. It has been documented that the development of early brain injury could be prevented by initiating autophagy under low-stress conditions [16,17]. The homeostasis roles of autophagy, as "remodeling" and "refreshment" functions, are critical for the differentiated cells, which has been well investigated in the neurons [13,18]. Based on these findings, we hypothesized that the intestinal catch-up growth of IUGR in response to colostrum during post-parturition period might be concerned with the intestinal autophagy. To test this hypothesis, a rat model of IUGR with catch-up growth was constructed by maternal 50% feed restriction as reported by Anderson et al.[19] and Desai et al.[20]. The intestinal structure, autophagy, apoptosis and proliferation were determined in this study. These results might be helpful to understand the responsible mechanism for intestinal catch-up growth of IUGR neonates in response to colostrum.

MATERIAL and METHODS

Ethical Procedures

Experiments were conducted under the supervision of the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China.

Animal and Experimental Design

IUGR rats were obtained from the rat model of IUGR as described by Anderson et al.[19] and Desai et al.[20]. Briefly, 20 primiparous Sprague Dawley rat dams, purchased from the experimental animal center of Soochow University (Jiangsu, China), were housed in the facilities with the relatively constant temperature of 20±2°C and a light-darkness cycle (12h:12h). From the gestational day 10 to 21, 20 dams were randomly assigned into two groups, which were provided with a commercial diet (crud protein: 20.19%; metabolic energy: 2.90 Mcal/kg) *ad libitum* (Adlib) and 50% feed restriction (FR) to achieve normal birth weight (NBW) and IUGR rat pups, respectively (n=10). The pups were weighed and litter size was recorded immediately following parturition. Eight pups were then kept in each litter to make sure that pups could have sufficient colostrum for 24 h. Subsequently, 2 pups (fast for 2 h) from each litter size were chosen and decapitated. The tail-vein blood glucose was analyzed with a glucometer (Bayer HealthCare, USA). The organs were weighed, including heart, liver, spleen, kidney, brain, and lung.

Analysis of Intestinal Histology

For the intestinal morphological evaluation, 1.5 cm-long jejunum samples (about 5 cm distance from pyloric sphincter) were obtained and analyzed as described by Dong et al.[8]. Briefly, the intestinal samples were fixed in 4% paraformaldehyde solution, dehydrated with graded series of ethanol and embedded in paraffin. The intestinal cross sections with 4 μm thickness were cut and stained with hematoxylin and eosin. Images of each section were obtained with an optical microscope (Olympus BX5, Olympus Optical Co. Ltd., Japan). Intestinal villus height, crypt depth and villus width were analyzed with an Image-Pro Plus 6.0 software. The villus height to crypt depth ratio (villi/crypt ratio) and villus surface area were calculated.

For the histological examinations with transmission electron microscope (TEM), 0.2 cm-long jejunum samples were fixed in 2.5% glutaraldehyde solution at 4°C for at least 48 h, post-fixed in 1% osmium tetroxide for 2h, dehydrated with series of ethanol, and finally embedded in EPOK. The TEM (Hitachi H-7500, Japan) was operated at 80 kV to observe the 70 nm thin sections, which were stained with uranyl acetate and Sato's lead staining solution [8].

Analysis of mRNA Expression

The analysis of mRNA expression in the jejunum of rat pups was conducted as described by Dong et al.[8] and Bustin et al.[21]. Briefly, approximate 8 cm jejunum samples (about 6.5 cm distance from pyloric sphincter) were quickly collected on ice, immediately frozen in liquid nitrogen and stored at -80°C. The total RNA was extracted with Trizol-based

procedure as recommended by the company (Invitrogen, USA). RNA quality and integrity was verified by agarose gel electrophoresis and by the determination of the absorption ratios of 260/280 nm and 260/230 nm (between 1.90 and 2.05) with Nano-drop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Subsequently, 1 µg RNA was used to obtain the cDNA with the Primer-Script™ reagent kit, which was purchased from TakaRa Biotechnology Co. Ltd. (Dalian, China).

The reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assay was performed on an ABI 7500 instrument (Applied Biosystems, Foster City, CA) using a SYBR Premix Ex Taq™ Kit (TakaRa Biotechnology Co. Ltd., Dalian, China) and specific primer sequences for the target genes, including *WIPI1, MAP1LC3B, Atg5, Atg14, Atg13, ULK1, mTOR, Beclin1, Bcl-2, Bax, caspase3, caspase9* and *ki67*. The primers for genes mentioned above were prepared by Invitrogen Biotech Co. Ltd. (Shanghai, China) and are listed in *Table 1*. After the primer specificity was checked with the tool of BLAST, the specificity was confirmed again by the experimental evidences (melting profile and electrophoresis gel). In addition, the efficiency of amplification was about 100% (95%-105%). The threshold values were determined with the ABI software and mRNA expressions were normalized to the reference-gene expression (*GAPDH*), which was stably expressed in rat jejunum as confirmed in present experiment. The relative mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ as described previously [22] and normorlized to the NBW pups.

Statistical Analysis

Data were analyzed by the student *t*-test of SPSS statistical package for Windows (IBM SPSS, version 20.0, Chicago) and expressed as mean ± standard error (SE). The litter was used as the experimental unit for the analysis of pup's body weight and litter size. For the other parameters, the individual rat pup was used as an experimental unit. The *P* value below 0.05 was considered as statistically significant level, and between 0.05 and 0.10 was considered as a tendency towards statistical difference.

Table 1. *Primers sequences used for real time PCR assays*

Genes	Accession No.	Primers	Sequences(5'--3')	bp
GAPDH	NM_017008.4	Forward	CAGGGCTGCCTTCTCTTGTG	170
		Reverse	TGGTGATGGGTTTCCCGTTG	
WIPI1	NM_001127297.1	Forward	CCAAGACTGCACATCCCTAGC	162
		Reverse	TGACTGACCACCACAACCAG	
MAP1LC3B	NM_022867.2	Forward	TCCTGAACCCCAGCCATTTC	141
		Reverse	GGCATGGACCAGAGAAGTCC	
Atg5	NM_001014250.1	Forward	CAGAAGCTGTTCCGTCCTGT	128
		Reverse	CCGTGAATCATCACCTGGCT	
Atg13	NM_001271212.1	Forward	AGGCTTCCAGACAGTTCGTG	118
		Reverse	TGGGGTCCTCTCAAATTGCC	
Atg14	NM_001107258.1	Forward	GGCTAACAGATCAGTTGCGATG	247
		Reverse	TGTTCCCTCAGGTCACTGGT	
mTOR	NM_019906.1	Forward	GCAATGGGCACGAGTTTGTT	94
		Reverse	AGTGTGTTCACCAGGCCAAA	
Beclin1	NM_053739.2	Forward	GCCTCTGAAACTGGACACGA	113
		Reverse	CTTCCTCCTGGCTCTCTCCT	
ULK1	NM_001108341.1	Forward	CATCCGAAGGTCAGGTAGCA	148
		Reverse	GATGGTTCCCACTTGGGGAGA	
Bcl-2	NM_016993.1	Forward	TCGCGACTTTGCAGAGATGT	116
		Reverse	CAATCCTCCCCCAGTTCACC	
Bax	NM_017059.2	Forward	GGGCCTTTTTGCTACAGGGT	106
		Reverse	TTCTTGGTGGATGCGTCCTG	
Caspase 3	NM_012922.2	Forward	GAGCTTGGAACGCGAAGAAA	221
		Reverse	TTGCGAGCTGACATTCCAGT	
Caspase 9	NM_031632.1	Forward	AGCATCACTGCTTCCCAGAC	328
		Reverse	CAGGTGTCCCCACTAGGGTA	
Ki67	NM_001108341.1	Forward	CATCCGAAGGTCAGGTAGCA	148
		Reverse	GATGGTTCCCACTTGGGGAGA	

RESULTS

Litter Size and Pup's Body Weight

Table 2 illustrates that maternal 50% feed restriction from gestation day 10 to 21 did not affect the litter size (P=0.68), however, it produced IUGR offsprings with decreased body weight (more than 10%) as compared with the Adlib dams (P<0.001).

Blood Glucose and Selected Organ Weights

Results indicated that there was no significant difference in blood glucose concentration and weights of spleen and brain between IUGR and NBW rats (P>0.05), as shown in *Table 3*. However, IUGR rats had significantly lower weights of heart, liver, kidney and lung as compared with the NBW rats (P<0.01).

Histological Observation of Jejunum

As shown in *Table 4*, compared to the NBW rats, IUGR rats had significantly reduced villus height (P<0.01) and villus surface area (P=0.012) and tended to have lower crypt depth (P=0.07). However, there were no significant differences in villi/crypt ratio, and villus width between IUGR and NBW pups (P>0.05). Moreover, IUGR pups showed accumulated autophagosomes in the jejunal mucosa *(Fig. 1A2, Fig. 1B2)*, however, the microvilli and mitochondrial histology were similar to the NBW pups *(Fig. 1A1, Fig. 1B1)*.

Autophagy Related Gene Expression

Effects of IUGR on the autophagy-related gene expression in the jejunum are presented in *Fig. 2*. Results showed that IUGR significantly enhanced mRNA expressions of *WIPI1*, *MAP1LC3B*, and *Atg13* (P<0.01), while expressions of *Atg5* and *Atg14* were unaffected in the jejunum (P>0.05). Compared with the NBW pups, IUGR pups had increased mRNA expressions of *ULK1* and *Beclin1*, and decreased *mTOR* mRNA expression (P<0.05).

Apoptosis and Proliferation Related Gene Expression

Effects of IUGR on expression of apoptosis and proliferation related genes in the jejunum are shown in *Fig. 3*. Results indicated that IUGR pups had significantly lower mRNA expression of *Bcl-2*, and higher *caspase 9* mRNA expression in the jejunum as compared to NBW pups (P<0.05). However, IUGR did not affect mRNA expressions of *Bax* and *caspase 3* (P>0.05), while it tended to increase *ki67* mRNA expression in the jejunum as compared with NBW pups (P=0.06).

Table 2. *The body weight of neonatal pups and litter size of rat dams*

Item[1]	NBW	IUGR	*P*
Litter size	13.90±1.79	13.50±2.37	0.68
Body weight (g)	6.42±0.08	5.77±0.08**	<0.001

*[1] Data were expressed as mean ± SE (n=10); ** P<0.01 means differences between IUGR and NW pups*

Table 3. *The blood glucose and selected organ weights of IUGR and NBW pups*

Item[1]	NBW	IUGR	*P*
Blood glucose (mmol/L)	6.52±0.37	5.69±0.34	0.11
Heart (g)	0.034±0.001	0.027±0.001**	<0.001
Liver (g)	0.317±0.010	0.253±0.013**	<0.001
Spleen(g)	0.010±0.001	0.010±0.001	0.451
Kidney (g)	0.064±0.002	0.055±0.002**	0.001
Brain (g)	0.214±0.005	0.221±0.005	0.276
Lung (g)	0.126±0.004	0.105±0.005**	0.001

*[1] Data were expressed as mean ± SE (n=20); ** P<0.01 means differences between IUGR and NW pups*

Table 4. *The intestinal morphology of IUGR and NBW pups*

Item[1]	NBW	IUGR	*P*
Villus height (μm)	260.49±6.95	227.63±6.55**	<0.01
Crypt depth (μm)	61.33±2.49	55.25±2.05	0.07
Villi/Cryptratio	4.41±0.17	4.20±0.13	0.34
Villus Width (μm)	58.36±1.28	57.32±1.12	0.55
Villus surface area (mm^2)	0.024±0.001	0.021±0.001*	0.012

*[1] Data were expressed as mean ± SE (n=20); * P<0.05 and ** P<0.01 means differences between IUGR and NW pups*

DISCUSSION

Maternal nutrition plays important roles in the development of fetuses as both the quality and quantity of maternal nutrients affect fetal development. Maternal malnutrition commonly causes low birth weight offsprings, those having birth weight less than 10th percentile are considered as IUGR. They commonly exert long-term effects on the health as termed "programming" [23-25]. Animals, such as pigs, sheep, and rodents, are widely used to construct IUGR models via maternal feed restriction [7,26,27]. Recently, it has been confirmed that rapid intestinal catch-up growth occurs in IUGR neonates which were provided with sufficient colostrum [12]. However, the underlying mechanism for catch-up growth is still largely unknown. Therefore, in this study, a rat model of IUGR with postnatal catch-up growth was constructed by maternal 50% feed restriction as previous reports [19,20].

In present study, maternal 50% feed restriction from gestational day 10 to 21 reduced neonatal body weight by more than 10%, which indicated that these pups were IUGR. These results were consistent with previous results reported by Desai et al.[20], who found that maternal feed restriction in late gestation decreased the body weights of offsprings, which showed rapid postnatal catch-up growth. Likely, Woodall et al.[28] reported that maternal

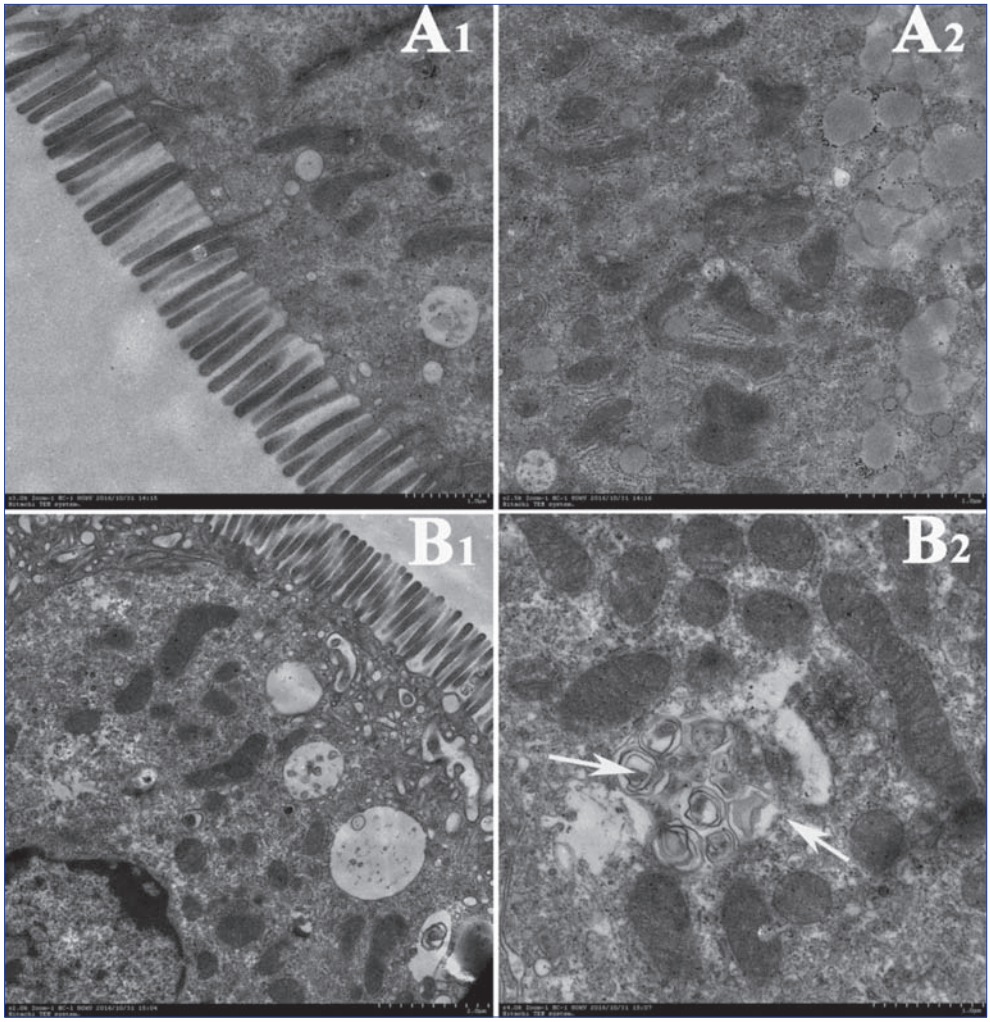
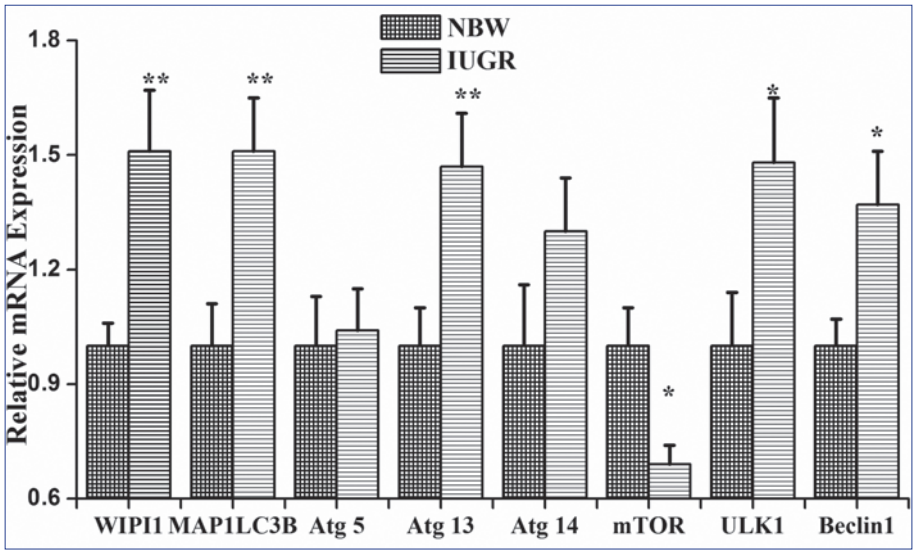


Fig 1. Effects of IUGR on the interior structure of the jejunum in pups. The microvilli of jejunum and its interior structure of NBW pups (A_1 and A_2) and IUGR pups (B_1 and B_2)

was not affected, which is in line with our present results that litter size was not affected by maternal 50% feed restriction from gestational day 10 to 21. Desai et al.[29] also found that maternal protein restriction throughout the pregnancy did not affect the litter size, but had selectively decreased organ growth. At the age of 21 days, the IUGR offsprings from protein restricted dams exhibited slight decreases in lung and brain weights, but a greater reduction in weights of pancreas, spleen, muscle and liver. In present study, IUGR pups had reduced organ weights, including heart, kidney and lung. However, Meyer et al.[30] and Anderson et al.[19] found that nutrient restriction during early to midgestation did not affect fetal organ weights, although the total and net maternal body weights were significantly decreased. The author suggested that dams could compartmentalize all the available nutrients to

Fig 2. Effects of IUGR on the autophagy related gene expression. No. for the expression was 10, data were normalized to the NBW pups and were expressed as mean± SE. * P<0.05 and ** P<0.01 means for the same parameter between IUGR and NBW pups were significantly different



feed restriction throughout the gestation produced IUGR pups, and inhibited the postnatal growth of the IUGR pups from birth to 90 days of age, while the litter size prevent serious fetal damages during maternal malnutrition [19], and sufficient maternal nutrients during the period of rapid fetal growth (the later gestation)

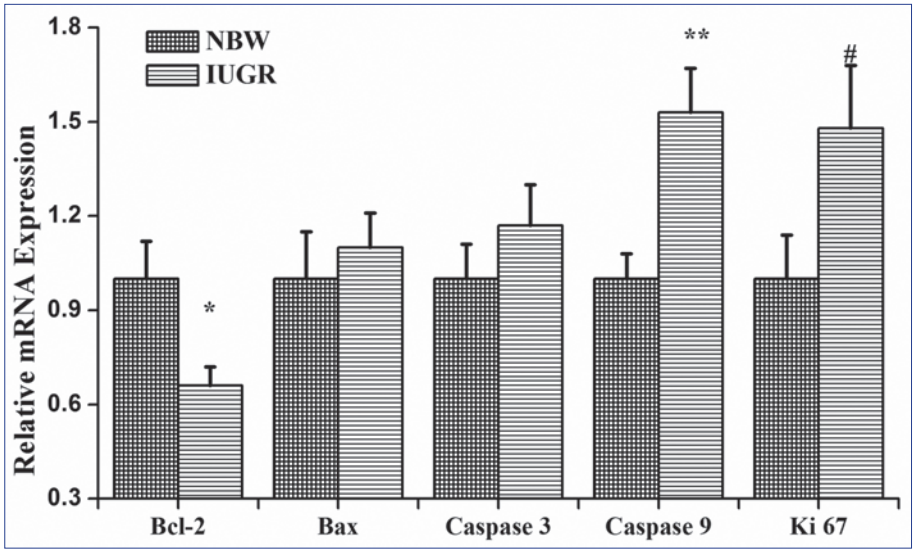


Fig 3. Effects of IUGR on expression of apoptosis and proliferation related genes in the jejunum. No. for the expression was 10, data were normalized to the NBW pups and were expressed as mean± SE. * P<0.05 and ** P<0.01 means for the same parameter between IUGR and NBW pups were significantly different, while # means a tendency towards significant difference (*P*=0.06)

are very important for the development of organs.

The small intestine plays a critical role in immunity, nutrient digestion and absorption. Various studies have focused on physiology and functions of small intestine of IUGR animals. For example, IUGR significantly lowered the small intestinal mucosa weight, decreased the length of the small intestine and colon in neonatal piglets [9], which was in line with previous reports [31]. Similarly, Dong et al.[8] observed that newborn IUGR piglets without suckling colostrum after birth had damaged and shorter intestinal villi with accumulated autophagosomes and swelled mitochondria in small intestine. Wang et al.[32] proved that the impaired small intestine of newborn IUGR piglets was accompanied with the altered intestinal proteomes. In agreement with previous reports, we found that IUGR pups showed decreased villus height and surface area, and tended to have lower crypt depth. However, microvilli and mitochondrial histology was unaffected in IUGR pups, indicating that sufficient colostrum could decrease the intestinal stress in IUGR pups, which is consistent with the previous report [12].

An accumulation of autophagosomes is an important indicator for the evaluation of autophagy [33]. In the current study, accumulated autophagosomes in the jejunum of IUGR pups were observed. Moreover, the IUGR pups also showed increased mRNA expressions of *WIPI1*, *MAP1LC3B*, and *Atg13* in the jejunum. It has been documented that the increase in mRNA expressions of *WIPI1* and *MAP1LC3B* is prior to the accumulation of autophagy marker protein *MAP1LC3* in a wide range of cells, suggests that analysis of their mRNA expression is one convenient method for monitoring autophagy [34]. Atg13 is in a complex with *Atg1*, *Atg101* and *ULK1*, which is essential for the induction of autophagy. Once the mRNA expression is decreased, the autophagy (in the cells of HEK293) is also reduced, which is similar to the *ULK1*depletion [35]. Therefore, these results indicated that IUGR enhanced the autophagy in the jejunum. To further elucidate the related molecular mechanism, the expression of *mTOR*, *Beclin1*, and *ULK1* in jejunum was determined in present study. It has been documented that *mTOR-Beclin1-ULK1* signal pathway plays critical roles in the regulation of autophagy [36-38]. As a sensor of nutritional status, stress and growth factor signals, *mTOR* can regulate autophagy through direct phosphorylation of *ULK1*, which further induces autophagy by phosphorylating *Beclin1* and activating *VPS34* lipid kinase [39,40]. Moreover, the up-regulated autophagy is commonly accompanied by the increased mRNA levels of *ULK1* and *Beclin1*, which plays critical role in embryonic development [34,41]. Results of present study showed that mRNA expressions of *Beclin1* and *ULK1* were increased, and *mTOR* gene expression was decreased in the jejunum of IUGR pups, which were in agreement with our results that the autophagy was enhanced in the jejunum, suggesting that the enhanced autophagy in jejunum of IUGR pups should be related with *mTOR-Beclin1-ULK1* signal pathway. However, the mechanism for the decreased *mTOR* mRNA expression in response to colostrum should be further studied as there was no significant difference in blood glucose, which is a primary energy source for intestinal development, between IUGR and NBW pups.

There is a complicated interaction among autophagy, apoptosis and proliferation. Despite the enhanced intestinal autophagy, IUGR pups still had higher mRNA expression of *caspase 9* and lower level of *Bcl-2* mRNA expression in present study, which indicated that intestinal apoptosis has been up-regulated [42,43]. The over-induced apoptosis might lead to further organ damage [44]. For example, Xia et al.[44] proposed that hypoxia induced renal autophagy via *Beclin1* signal pathway, enhanced apoptosis and affected the renal development in IUGR rat fetuses. We also observed the similar phenomenon in small intestine of IUGR fetuses (data not published) and newborn IUGR piglets (without feeding colostrum) that over-enhanced autophagy and apoptosis might

further impair the intestinal morphology [8]. However, after feeding with sufficient colostrum for 24 h, IUGR pups were under low-stress conditions as discussed above, but still exhibited increased intestinal autophagy in this study, which might contribute to the rapid catch-up intestinal growth [12,16]. It has been documented that autophagy regulates differentiation via notch signaling pathway [45]. Therefore, enhanced intestinal autophagy under low-stress conditions after feeding with sufficient colostrum may enhance the proliferation of intestinal crypt base columnar stem cells and improve the crypt regeneration [46,47]. In consistence with these results, the *ki67* mRNA expression tended to increase in the jejunum of IUGR pups fed with colostrum in present study. The mRNA expression of *ki67* is a sensitive indicator for the proliferative status. Once the cell exits from the active cell cycles, the reduced *ki67* mRNA expression can be easily detected [48,49]. Specific reduction of *ki67* mRNA inhibits the proliferation and increases apoptotic cell death [49]. Therefore, the tendency towards increased mRNA expression of *ki67* in present study suggested that the intestinal proliferation in IUGR pups fed with colostrum tended to be enhanced, which may contribute positively to the rapid intestinal catch-up growth. In accordance with our present results, we also found that IUGR pups with sufficient colostrum/milk had similar villus height and crypt depth, and tended to increase the villus width and surface area as compared to the NBW rat pups at the age of 7 days (data not published). Similarly, previous studies also verified that under low-stress conditions enhanced autophagy could prevent early brain injury [16,17].

In summary, our present results indicated that maternal 50% feed restriction from gestational day 10 to 21 did not affect the litter size, but produced IUGR pups. After feeding with sufficient colostrum for 24h, IUGR rat pups still had impaired intestinal morphology with enhanced apoptosis and increased autophagy via *mTOR-Beclin1-ULK1* signalling pathway. However, the small intestine of IUGR pups with sufficient colostrum was under low-stress conditions (unaffected intestinal mucosal mitochondrial histology). Combination with previous reports that autophagy (under low-stress conditions) could enhance proliferation and our results (the tendency to increase *ki67* mRNA and the catch-up growth in intestinal morphology at day 7), it can be concluded that the rapid intestinal catch-up growth of IUGR pups in response to sufficient colostrum should be related to the enhanced intestinal autophagy (under low-stress conditions) and intestinal proliferation. These results may be beneficial for the development of IUGR neonates during post-parturition periods. However, the long-term effects of catch-up growth on the health should be investigated precisely in future.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Research Article

Association Study Between Mannose-Binding Lectin *(MBL)* Polymorphisms and Serum *MBL* Protein Levels After *Mycoplasma ovipneumoniae* Infection in Sheep

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Abstract

Mannose Binding Lectin (*MBL*) is a liver-derived, circulating plasma protein that plays a pivotal role in innate immunity, however, *MBL* polymorphisms in sheep were associated with the incidence of *MO* infection. The objective of this study was to determine the relationships between the different genotypes of *MBL* and the resistance against *MO* infection disease, including three hundred and thirty-six individuals of the China Merino sheep. Single-Strand Conformation Polymorphism (SSCP) analyses of PCR amplicons of the exon-1 region of the *MBL* gene revealed four patterns: BB, BC, CC and DD. However, BB, BC and DD genotype have 3 SNPs, 2 SNPs, and 4 SNPs respectively, and CC genotype has only an identical sequence to the reference, among which there are 3 synonymous SNPs and 6 non-synonymous SNPs. Our statistic analysis showed that the DD genotype *MBLs* in China Merino sheep were associated with the decrease of *MBL* protein level in serum. The sheep carrying this kind of genotype were more susceptible to *MO* infection, however, an opposite result was found in sheep having CC genotype, which will provide a reference for molecular breeding of sheep breeds resistant to *MO* infection.

Keywords: *Polymorphisms, China Merino sheep, Mannose-binding lectin, PCR-SSCP, Mycoplasma ovipneumoniae infection*

Koyunlarda Mannoz Bağlayan Lektin *(MBL)* Polimorfizmi ve *Mycoplasma ovipneumoniae* Enfeksiyonu Sonrasında Serum *MBL* Protein Seviyeleri İle İlişkisi

Özet

Mannoz Bağlayan Lektin *(MBL)* karaciğer kaynaklı, dolaşımda yer alan plazma protein olup doğal bağışıklıkta önemli bir rol oynar. Koyunlarda *MBL* polimorfizmi *Mycoplasma ovipneumoniae (MO)* enfeksiyonu ile ilgilidir. Bu çalışmanın amacı 136 Çin Merinos koyununda farklı MBL genotipleri ile *MO* enfeksiyonuna karşı direnç arasındaki ilişkiyi araştırmaktır. *MBL* geninin ekzon-1 bölgesinin PCR amplikonlarının Tek Zincir Konformasyon Polimorfizm (SSCP) analizi BB, BC, CC ve DD olmak üzere dört şekil bulunduğunu tespit etti. BB, BC ve DD genotipleri sırasıyla 3 SNP, 2 SNP ve 4 SNP'ye sahipti. CC genotipi referans ile aynı bir sekansa sahip olup aralarında 3 sinonim SNP ve 6 sinonim olmayan SNP vardı. İstatistiksel analizler, Çin Merinos koyunlarında DD genotip MBL'nin serumda azalmış *MBL* protein seviyesi ile ilişkili olduğunu gösterdi. Bu tip bir genotipe sahip olan koyunlar *MO* enfeksiyonuna daha duyarlıyken CC genotipe sahip olanlarda tam tersine bir görüntü elde edildi. Bu durum *MO* enfeksiyonuna dirençli koyunların elde edilmesi için bir referans oluşturmaktadır.

Anahtar sözcükler: *Polimorfizm, Çin Merinos koyunu, Mannoz bağlayan lektin, PCR-SSCP, Mycoplasma ovipneumoniae enfeksiyonu*

INTRODUCTION

Mannan-binding lectin *(MBL)* is a member of C-type lectin superfamily lectins collagen family, which can selectively identify Mannan, N an acetyl Glucosamine and mannose, N an acetyl mannosamine, etc. *MBL* can bind to a variety of bacteria, viruses, fungi, *Mycoplasma*, parasites, etc. It is an important part of anti-inflammatory immune response

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and constitutes the first defense line against infection by activating complement and opsonophagocytosis [1].

Serum *MBL* level and *MBL* gene mutation are closely associated with many kinds of diseases. *The MBL - defect* could result in many kinds of acute or chronic pathogen infections. Abnormal serum *MBL* levels might also cause certain autoimmune diseases, the mutations in exon-1 of the human *MBL* gene can interfere the formation of a stable *MBL* functional polymer which decreased serum *MBL* levels and lowered the body's physiological function significantly, such as *Systemic lupus erythematosus*, *Clinical repeated infections*, *Hepatitis B virus*, and *other viral infections* in humans [2-5]; Similarly, some other studies also reported that A-type *MBL* can has antibacterial function against *Porcine Actinobacillus*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* [6,7]. It showed that *MBL* plays a vital role in the activation of the complement pathway as anti-infective molecules.

Recently, with the increase in breeding stock and changes of the introduction and feeding methods in sheep in Xinjiang, China, the risk of subsequent propagation of *MO* explosive increased and seriously affected the sustainable and healthy development of sheep. Especially, China Merino sheep, as one of the most famous kinds of fine wool sheep in Xinjiang, has been infected seriously which strongly threatened the wool industry. Therefore, in order to expand theoretical and practical knowledge of *MBL* resistance to *MO* and other relevant diseases, it is necessary to study the relationship between *MBL* genotypes and anti-*MO* infection in sheep [8,9]. Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) technology in this study was used to explore the correlations between different *MBL* genotypes and serum *MBL* levels in sheep, which will provide a reference for sheep breeding and genetic research of immune disease in future.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University (Number: A2010096). All samples were collected in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Experimental Animals and Mycoplasma

A total of 336 healthy individuals of the China Merino sheep that aged 3 months and weighted 8-12 kg were from different sheep farms in the ninth agricultural unit of the Xinjiang Production and Construction Corps, China. A strains of *Mycoplasma (Named MO-141)* was isolated from *Hu Sheep* with *MO* infection in shihezi, Xinjiang, which the DNA homology of *MO-141* and standard strain Y-98 reaches 99% by sequence alignment, and then the isolated strain was inoculated with *Mycoplasma* liquid enrichment medium, which were incubated for 7 days under 5% CO_2 at 37ºC condition. After the culture medium turned yellow, it was put in centrifuge that were separated by centrifugation at 20.000 r/min for 20 min at 4ºC condition, when bacterial counts were 10^6 CCU/mL and then used to construct a sheep model with *MO* infection. And then, the experimental groups were injected with *MO* into the trachea 5 mL/each with a syringe, the control group was injected with the same amount of sterile saline trachea.

Collection of Sheep Blood

It selected thirty-six China Merino sheep with four different *MBL* genotypes by using PCR-SSCP technology, which were averaged into four groups, including BB, BC, CC and DD genotypes respectively, and then were marked as No. 1-36, and then the control group was numbered 37-42. Sheep infected with *MO* in the experimental group, which were collected the fresh anticoagulant blood on the day before infection (-1 d), and 1 d, 7 d, 14 d and 21 d after artificial infection respectively. Meanwhile, the fresh anticoagulant blood in the control group were also collected and stored at -20ºC.

Sheep Genomic DNA Extraction, Primer Design and PCR Amplification

Sheep genomic DNA was extracted from whole blood samples of the China Merino sheep using the phenol/chloroform method as described in Sambrook and Russell (2001). Two primers, including *MBL*F (5-CGCTGTTTACAT CACTTCCT-3) and *MBL*R (5-CACTGTACCTGGTTCTCCCT-3), were designed using Primer 5.0 from the sequences of the *MBL* gene of sheep available in GenBank (accession numbers FJ977629). Two Primers were synthesized at Sangon Biological Engineering Technology Company (SBETC, Shanghai, China) and used in 25 μL PCR reaction to amplify a 194 bp section of the exon-1 region of the *MBL* gene. A total volume of 25 μL PCR reaction contains 1 μL (50 ng) of genomic DNA extracted from an individual China Merino sheep, 2.5 μL 10× PCR buffer, 1 μL (10 mM) of each primer, 2.5 μL dNTPs (2.5 mM), 1.5 μL $MgCl_2$ (15 mM), 0.6 μL (2.5 units) Taq DNA polymerase, and 14.9 μL MilliQ H_2O. The PCR reagents were supplied by the SBETC. The procedure for PCR reactions are 94ºC denaturation for 30 s, annealing for 45 s, 72ºC extension for 30 s, 35 cycles, final extension at 72ºC for 10 min. PCR products were detected by 1.5% agarose gel electrophoresis.

Cloning of PCR Products and DNA Sequencing

PCR products were analyzed by SSCP, aliquots of 2 μL PCR products were mixed with 8 μL denaturing solution (98% formamide, 25 mL MEDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98ºC for 10 min and then chilled on ice for 10 min. Denatured PCR products were run on 12% PAGE gel (80 mm ×73 mm ×

0.75 mm) in 0.5 ×TBE buffer at 140 V and 12°C for 20 h. The gel was stained with 0.1% silver nitrate solution. According to the result of PCR-SSCP, PCR products containing the single nucleotide polymorphisms (SNPs) site that was recovered by Omega Gel Extraction Kit, connected with the pMD19-T vector, transformed into DH5α competent cells, picked clones, shaked bacteria, and then identified by bacteria PCR. Lastly, the homozygous genotype was send to sequence at BGI (Beijing, China; http://www.genomics.cn).

Measurement of Serum MBL Levels in Sheep

Sheep *MBL* levels in serum were measured using the *MBL* Oligomer ELISA Kit (ADL, America) in the experimental group and the control group before and after artificial infection. Serum samples from the China Merino sheep and standards of known *MBL* concentrations were loaded into 96 wells on the test plate, and then the *MBL* antigen and the biotinylated monoclonal antibody specific to *MBL* were added to each well and were incubated at 37°C for 60 min. The wells were washed and the enzyme, streptavidin-peroxidase, was added. After incubation at 37°C for 30 min, the wells were washed to remove unbound enzymes, and the substrate solution was reacted with the bound enzyme to induce a colour. The intensity of the colour was proportional to the concentration of *MBL* protein present in the serum samples, which was measured with an ELISA reader at 450 nm and then converted into *MBL* concentration (μg/L) in serum.

Statistical Analysis

Differences in haplotype frequencies were analyzed using a χ2-test. The association between polymorphisms in *MBL* gene and *MBL* protein levels in serum were evaluated using One-Way ANOVA test. All statistical analyses were performed with SPSS for Windows (version 19.0).

RESULTS

Polymorphisms in MBL Gene in China Merino Sheep

SSCP analyses of the PCR-amplified fragments in our study from the 194-bp section of the exon-1 of the *MBL* gene showed four distinct banding patterns in China Merino sheep (n = 336), including BB, CC, DD and BC *(Fig. 1)*. We used the sequences of the full-length *MBL* gene of sheep available in GenBank (accession numbers FJ977629) as a reference to compare with the sequences we obtained from China Merino sheep. FJ977629 has identical sequences for the exon-1 region of the *MBL* gene. Our comparisons showed that pattern CC had an identical sequence to the reference, whereas the other three patterns had sequence variation from the reference. Pattern BB and DD had four mutation sites, respectively: one at position 105 that was synonymous, and the other three at position 28, 43 and 86 that were non-synonymous, which resulted in three amino acid changes in the putative *MBL* protein (Leu 10 Phe, Met 15 Val and Thr 29 Ser). Pattern BC had two SNPs: one at position 105 that was synonymous, and the other at position 43 that was non-synonymous, which resulted in amino acid changes in the putative *MBL* protein (Met 15 Val) *(Fig. 2)*.

Association Between Polymorphisms in MBL Gene and MBL Protein Levels in Serum in China Merino Sheep

The serum *MBL* levels of the 42 China Merino sheep were determined before *MO* infection by Sheep *MBL* ELISA kit, and then the results showed that serum *MBL* levels with CC-type was the highest, followed by subsequently BB-, BC- and DD-type, based on which predicted that CC genotype would be as a resistance group and DD genotype was susceptible *(Fig. 3)*. However, the serum *MBL* levels were determined on the 21th day after artificial *MO* infection that the mean values of serum *MBL* levels of 4 genotypes were lower than the control group, among which the rate of decline in CC and BC were bigger than BB and DD *(Fig. 4)*.

Meanwhile, the antibody OD values were detected between the experimental group and the control group before artificially infected with *MO* (OD >2NC + 0.06 was positive), which the results showed that

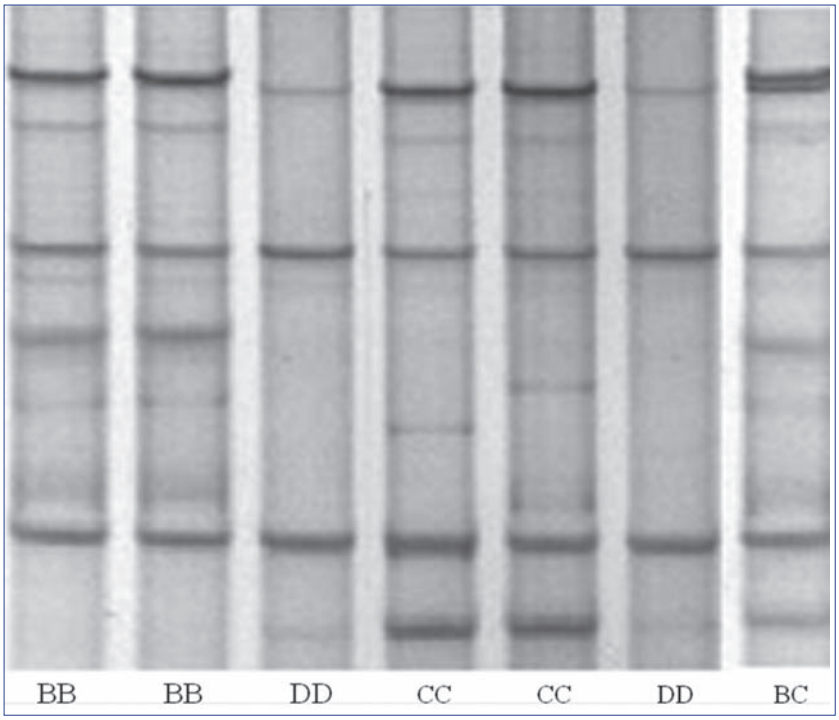

Fig 1. Electrophoretic patterns of PCR-SSCP of *MBL* exon 1 in *Chinese Merino sheep*

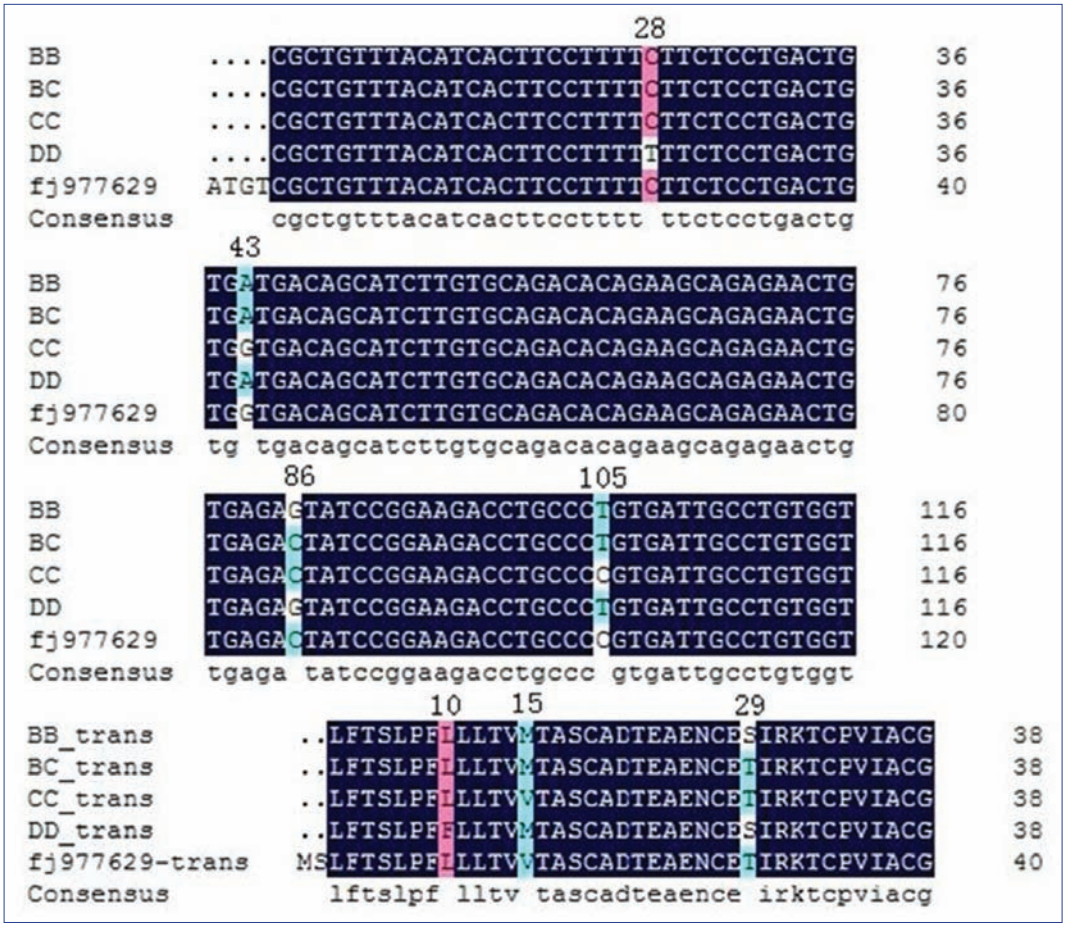

Fig 2. Comparison of the results of *MBL* exon1 polymorphism sequencing and amino acid substitutions in *Chinese Merino Sheep*

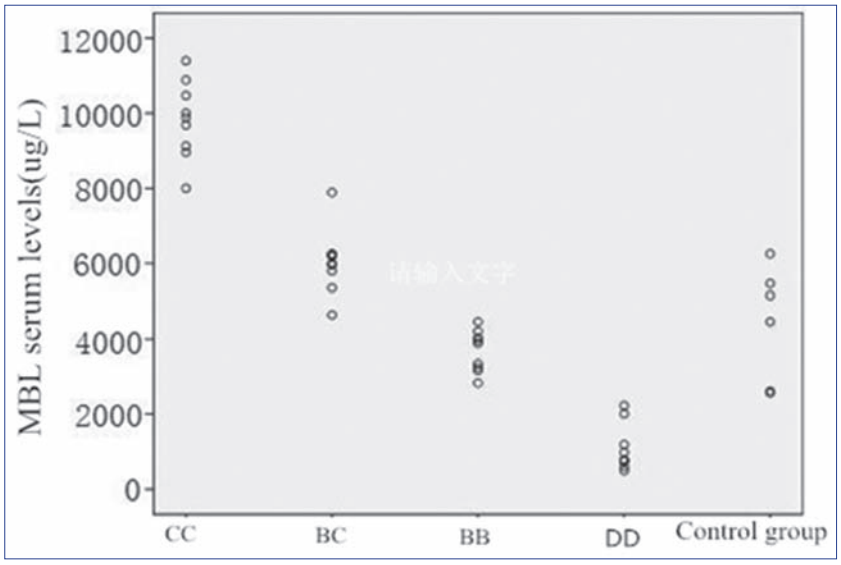

Fig 3. Comparation of *MBL* serum concentration in different genotypes of *MBL*-exon1 before infection

all individuals were negative before artificial infection. However, after *MO* artificial infection, all individuals in the control group were negative, and one half of the individuals in the experimental group were positive. On the 57th day after artificial *MO* infection, there were six sheep died in the experimental group, and then were dissected to carry out pathological findings. Later, all the rest of sheep were determined by ELISA antibody kit, the results showed that there were 2 ill-sheep in DD-type group, 7 ill-sheep in CC-type group, 4 ill-sheep in BC-type group, and 5 ill-sheep in BB-type group, however, all individuals in the control group were not ill *(Table 1)*. Fisher exact statistical analysis showed that the rate of *MO* infection in the DD-type group was significantly lower than the CC-type group ($P<0.05$).

DISCUSSION

Mannan-binding lectin, an important natural anti-infective immune molecule in humans and animals, which is secreted into the blood after synthesis by the liver and induces and activates

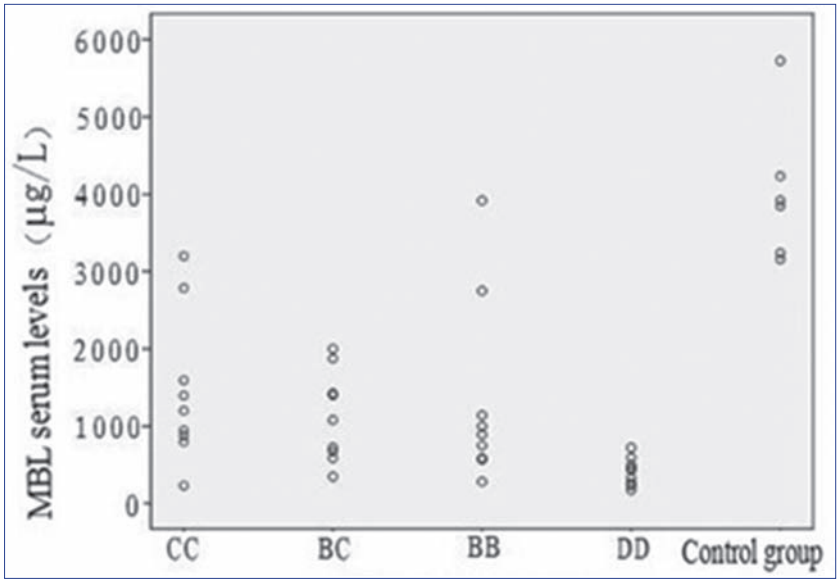

Fig 4. Comparation of serum *MBL* concentration in different genotypes of *MBL*-exon1 after infection

decrease in the serum concentration of *MBL*. Point mutations in exon-1 of the human *MBL* gene are frequently described as being associated with *MBL* plasma concentration, reduced ligand-binding capacity and failure to activate complement [15]. The exon-1 mutations on the protein product are believed to impair oligomer-ization and lead to a functional deficiency. Most mammalian species, including pigs, have 2 forms of *MBL* (A and C) [6,16-18]. Low expression of *MBL-C* was observed in most diseased animals almost all of which had one or both mutations, whereas clinically healthy pigs had a wide range of *MBL-C* expression. As a report that Heterozygotes have reduced serum concentrations of *MBL*, whilst functional multimeric protein is almost absent from the serum of homozygotes

Table 1. *Results of artificial infection with MO in different genotype individuals in Chinese Merino sheep*

Sheep No. Genotypes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
DD (susceptible group)	+	+	+	+	+	+	-	+	-
CC (resistant group)	+	-	-	-	-	-	-	+	-
BC	-	+	+	+	-	-	+	-	-
BB	-	+	+	+	-	-	+	+	-
Control group	-								

Numbers 1-9 just represent the number of sheep in each group. + represents incidence and - no incidence

the body's immune response before the antigen-specific antibody reacts specifically. Functional MBL is a multimeric protein of up to six 96 kDa subunits, which consisted of 3 identical polypeptide chains pro-duced by the liver. There are two pathways by which *MBL* can participate in a host defense response: 1) *MBL* activates the lectin complement pathway via *MBL* associated serine proteases *(MASPs),* that con-verges with the classical complement pathway, at the level of complement C4, and 2) MBL may also act directly as an opsonin, enhancing phagocytosis by binding to cell-surface receptors present on phago-cytic cells [5,6]. Several reports suggest that *MBL* deficiency also predisposes to autoimmune disease, such as systemic lupus erythematosus [10,11], and rheumatoid arthritis [12]. *MBL* deficiency is associated with an increased susceptibility to infection with *Neisseria meningitidis* [13], and severity of atherosclerotic disease [14]. *MBL* binding may facilitate the uptake of *Mycobacterium* by macrophages, thereby promoting infection.

Polymorphisms in the *MBL* promoter have been shown to be associated with the prevalence of infectious diseases. The point mutations, three in exon-1 and two in the promoter region of the *MBL* gene, lead to a dramatic and compound heterozygotes [19]. Similarly, it is also shown in our study these polymorphisms disrupt the assembly of *MBL* peptide trimers or accelerate the *MBL* degradation, and result in profoundly reduced serum levels of functional polymeric *MBL*.

To verify the relationships between the *MBL* genetic polymorphisms and anti-*MO* infection in China Merino sheep that were firstly divided into four types and then different *MBL* genotypes of sheep were artificially infected with *MO*. The results showed that a model of artificial infection of *MO* was successfully established that was used to study the correlation between polymorphisms in *MBL* genotypes and serum *MBL* protein levels in China Merino sheep. Meanwhile, *MO*-infected individuals showed that there were very obviously clinical symptoms, such as a lot of pleural effusion, pulmonary and pleural with cellulose pigmentation, a section of the pneumonia area with status marmoratus and other pathological features. Thus, it revealed that *Mycoplasma* was successfully isolated from the lungs in a sheep model with *MO* infection.

It is worth mentioning that here, Hamvas et al.[20] study showed that there were two thirds of the patients with

Mycoplasma infection accompanied by *MBL* deficiency, whereas there were only one third of the healthy individuals accompanied by *MBL* deficiency, which showed that *MBL* plays an important role in preventing *Mycoplasma* infection and there might a positive correlation between the susceptibility individuals and exon-1 mutations in *MBL*. Interestingly, in our study, there were 4 single-base nucleotide mutations in Exon-1 of *MBL* gene in China Merino sheep, including g.28C > T, g.43A > G, g.86G > C and g.105T > C by using the PCR-SSCP technology to analyze the genetic polymorphism of the exon-1. Of which, g.28C > T, g.43A > G and g.86G > C were non-synonymous respectively, causing the amino acid replacement of Leu10Phe, Met15Val, and Thr29Ser, however, g.105T > C was synonymous. Exon-1 encodes the signal peptide sequence that has cysteine-rich region and eight repeating Gly-X-Y motif. The present study showed that different mutations in exon-1 were located within the first 28 amino acids, which might affect a signal peptide synthesis and hinder the synthesis of the polypeptide chain. At the same time, these mutations in the promoter region of *MBL* gene were thought to reduce *MBL* expression by impairing the binding of transcription factors. The exon-1 mutations in the *MBL* gene were believed to impede the assembly of *MBL* subunits into the basic trimer structure, thereby reducing the amount of functional *MBL* subunits in heterozygous individuals [21,22]. Similarly, a study also showed that polymorphisms in exon 1 of the *MBL*-2 gene were significantly overrepresented in individuals with primary antibody deficiency and culture-proven *Mycoplasma* infections which could result in reducing plasma levels of *MBL* [23]. Therefore, this study further analyzed the relationship between *MBL* exon-1 polymorphisms and serum *MBL* levels in China Merino sheep with *MO* infection. The results in our study showed that serum *MBL* levels of CC-type were significantly higher than DD type in exon-1, which forecasted that CC-type was related to the resistance of sheep with *MO*. In addition, another artificial infection experiment also confirmed that CC-type in China Merino sheep had significant resistance compared with DD-type [24]. Single locus association analysis showed that g.86 G > C loci DD genotype in China Merino sheep that was significantly correlated *MO* and the infection rate was 78%, one explanation which could be that serum *MBL* levels was lower that caused immune dysfunctions to subject to diseases of various kinds, or *MBL* mutant sites might be involved in mediating some cells in *vivo* that were favorable for *Mycoplasma* invasion to make these individuals were more susceptible to *MO*. Thus, g.86G > C loci in exon-1 region of *MBL* can be used as molecular markers for *MO* susceptibility in sheep.

In summary, considering the results obtained here, we can conclude that the exon-1 of *MBL* gene polymorphisms influence *MBL* serum levels in China Merino sheep, which there were four polymorphisms in the exon-1 of the *MBL* gene in China Merino sheep that were closely related to resistance to *MO* of China Merino sheep. More importantly, it could be used as a molecular marker of resistance breeding in sheep that would provide a reference for further studying molecular mechanism of *MBL* gene in livestock breeding for disease resistance.

Competing Interests

There are no potential conflicts of interest.

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Research Article

Effect of ZnO Nanoparticles on *In Vitro* Gas Production of Some Animal and Plant Protein Sources

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Abstract

This study was conducted to determine effect of adding ZnO nanoparticles at levels of 0, 30 and 60 ppm on *in vitro* gas production of some animal and plant protein sources. In this study, gas production at 2, 6, 12, 24, 48 and 72 h incubation were measured and 200 mg of samples were used for gas production analysis. The results showed that after 72 h of incubation, the most volume of gas production in the plant protein sources of soybean meal (SM) and in between the sources of animal protein in poultry offal meal (POM) were respectively 58.23 and 28.34 mL per 200 mg of dry matter was obtained. In related with the parameters of nutrition from incubation data, metabolizable energy (ME), for soybean meal at the levels of zero, 30 and 60 ppm ZnO nanoparticles added to the 8.55, 8.81 and 7.54 were highest and for blood meal (BM) were lowest 2.26, 2.31 and 2.01 MJ/kg dry matter (DM), respectively. The highest and the lowest amount of organic matter digestibility (DOM), short-chain fatty acids (SCFA) and microbial protein (MP) were also for SM and BM. Overall, the results showed that using levels of 0, 30 and 60 ppm of ZnO nanoparticles was no effect on *in vitro* gas production of some animal and plant protein sources but had no significant effect in some hours of incubation, gas production and nutrition parameters.

Keywords: *In vitro gas production, Nutrition parameters, Protein sources, ZnO nanoparticles*

ZnO Nanopartiküllerinin Bazı Hayvan ve Bitki Protein Kaynaklarının *İn Vitro* Gaz Üretimi Üzerine Etkisi

Özet

Bu çalışma bazı hayvan ve bitki protein kaynaklarının *in vitro* gaz üretimi üzerine 0, 30 ve 60 ppm düzeylerinde ZnO ilavesinin etkilerini belirlemek amacıyla yapılmıştır. Çalışmada 2, 6, 12, 24, 48 ve 72 saat inkübasyolarda gaz üretimi ölçülmüş ve gaz üretim analizi amacıyla 200 mg örnek kullanılmıştır. 72 saat inkübasyon sonrasında bitki kaynaklarından soya fasulyesi yemi (SM) ile hayvan kaynaklarından kanatlı sakatat yeminden (POM) elde edilen en fazla gaz üretimi 200 mg kuru maddede sırasıyla 58.23 ve 28.34 mL olarak belirlendi. İnkübasyon verilerinden elde edilen gıda parametrelerinde, soya fasulyesi yemi için metabolize edilebilir enerji 0, 30 ve 60 ppm ZnO nanopartikül ilavelerinde sırasıyla 8.55, 8.81 ve 7.54 olup en yüksek seviyede ve kanlı yem (BM) için en düşük seviyede olup sırasıyla 2.26, 2.31 ve 2.01 MJ/kg kuru madde (DM) olarak tespit edildi. En yüksek sindirilebilir organik madde (DOM) miktarı, kısa zincirli yağ asitleri (SCFA) ve mikrobiyal protein (MP) SM için belirlenirken en düşük seviyeler BM için tespit edildi. Sonuç olarak; 0, 30 ve 60 ppm düzeylerinde ZnO nanopartiküllerinin kullanımının bazı hayvansal ve bitkisel kaynaklarda *in vitro* gaz üretimi üzerine etkisinin olmadığı ve inkübasyon süreleri ile besin parametreleri üzerine anlamlı bir etkisinin bulunmadığı belirlenmiştir.

Anahtar sözcükler: *İn vitro gaz üretimi, Besin parametreleri, Protein kaynakları, ZnO nanopartikülleri*

INTRODUCTION

Zinc is as an essential trace element for almost all living organisms. This element is vital for the functionality of more than 300 enzymes and other metabolic functions such as transcription RNA, defense against free radicals and replication of DNA [1], and this is due to that zinc should be added the daily diet of ruminants [2] and for ruminant nutrition and their rumen microorganism is necessary [3]. Intake of zinc by ruminants with their rumen microbial population changes resulted in changing of the ruminal digestion and fermentation process [4].

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Addition Zn to ruminant diet to more than needed, change their rumen fermentation and increase the ratio of propionic acid and decreases acetate to propionate ratio and it is concluded that increases the energy value of the diet [5]. Because of the unique characteristics of ZnO nanoparticles, these materials used in various industries including food, pharmaceutical, rubber, electronics and packaging and even as feed additives [6]. Reduction of particle size in the nano-scale has led to increased contact area the combination with other biomolecules and these organic molecules and inorganic chemical reactions in the body can be very different that in many materials is still unknown [7]. In relation to the effects of ZnO nanoparticles in biological systems and particularly bacteria, many researches have been done by other researchers such as antimicrobial effects of the substance on the bacteria *Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermises* have confirmed [8]. As for the effect of ZnO nanoparticles is very little research has been done on the performance of livestock and poultry. In a study, the use of ZnO nanoparticles at 40 mg per kg diet DM, improved poultry performancedue to essential role for body's appropriate physiological functions specially functional enzyme [9].

The *in-vitro* gas production technique have been usually used to assess feed evaluation for ruminants [10-12]. Advantages and disadvantages of *in-vitro* gas procedure are debated by Gatechew *et al*.[13]. A simple *in vitro* methodology is designated by Menke *et al*.[14] which is useful and fast, and permits a large number of samples to be ran at a stage. Makkar [15] highpoints the potential of a novel methodology using an *in-vitro* gas prodution techniques for evaluation of nutritional quality of feed resources. Recently, *in-vitro* gas production technique for feed evaluation well considered by Singh *et al.*[16]; Ayaşan *et al.*[17]; Ayaşan *et al.*[18] and Sevim *et al*.[19]. As a result, *in vitro* gas production technique is used as potentially useful technique to estimate feed intake, organic matter (OM) and dry matter digestibility, metabolizable energy (ME) of feeds and ruminal fermentation studies for ruminants [20-24]. Manipulation of ruminal fermentation with meet the needs of mineral elements for rumen microorganisms, particularly minerals such as Zinc (nano form) may be improves efficiency of protein metabolism [25].

In an experiment [26] of 0, 10 and 20 mg of zinc in ml rumen fluid *(in vitro)* used and its effect on rumen fermentation evaluated after 24 h of incubation and observed that the ruminal pH and ammonia levels were not affected by the zinc levels. Also, in an *in vitro* test using ZnO nanoparticles in the diet improve rumen bacterial growth and increasing the efficiency of energy intake in the diet [27]. In other experiment [28] amount of *in vitro* gas produced over 144 h of incubation there was no significant difference in treatments containing zinc at levels 20 and 40 ppm with control. As mentioned above, with the development of nanoparticles and its use in industries as well as due to the possibility of using it in feed livestock industry and insufficient data, however, little was known about influence of ZnO nanoparticles on ruminant nutrition, this experiment was designed. Thus, the objectives of this study were to evaluate effect of adding ZnO nanoparticles at levels of 0, 30 and 60 ppm on *in vitro* gas production of some animal and plant protein sources.

MATERIAL and METHODS

Materials

Nano-ZnO was purchased from Iranian agent of US Research Nanomaterial, Inc. Port Co., Ltd., USA. The sizes of elemental ZnO particles ranged from 10 to 30 nm, stock: US3590, in the form of white powder and Purity: 99%, APS: 10-30 nm, Color: white, Crystal Phase: single crystal, Morphology: nearly spherical, SSA: 20-60 m2/g, True Density: 5.606 g/cm3.

Methods

Sample Preparation, Chemical Analysis and in vitro Digestibility

This experiment was conducted at the Animal Science Laboratory of Mohaghegh Ardebili University in Iran. This experiment was conducted on sources of plant protein (SM, rapeseed meal, RM; and cottonseed meal, CM and sources of animal protein (POM, fish meal, FM and BM). The samples of SM, RM, CM, POM, FM and BM studied were obtained from feed compound manufacturers, the agricultural sector and the local slaughter house of North West Iran Ardebil Province (Meshgin, Germi and Ardabil), over the years 2014 and 2016. The prepared samples from local factories, for preventing degradation and degreasing, were used carrier materials or moisture adsorbent such as wheat bran. Therefore, some of its analyzes did not match to world feed standard analysis and their cell wall values were higher. The samples were randomly selected for the survey. Then, two local associations were randomly selected from each of the famous regions. A systematic sampling was done in each of the selected associations until total fifteen farmers or agricultural sector were selected for the study, which brings the number of farmers selected to thirty in every region. The chemical composition of the feed by conventional methods [29] and determination of *in vitro* digestibility was estimated using the equation described by Menke et al.[14]. Subsamples of protein sources were grounded through a 1 mm screen and defatting was done by extraction with petroleum ether for 6 h according to the AOAC procedure [29]. Samples of feeds were dried in a forced-air oven at 65-70ºC for24 h and DM content calculated. Ground samples (1 mm) were analysed for ash (ID 942.05) [29] and Kjeldahl N (ID 954.01) [29]. Crude protein (CP) was calculated as Kjeldahl N×6.25. Neutral-detergent fibre (NDF), acid-detergent fibre (ADF)

and acid-detergent lignin (ADL) were determined by the detergent procedures of Van Soest [30,31], with alpha amylase being added during NDF extraction. Sodium sulfite was not added. NDF was expressed without residual ash. Ether extract (EE) was determined by extracting the sample with petroleum ether using a Gerhardt Soxtherm 2000 Automatic (ID 920.39) [29].

In vitro Gas Production

Incubation was carried out at 39°C and the volume of gas production was measured at 2, 6, 12, 24, 48 and 72 h using procedures described by Menke and Steinggass [10]. Approximately, 200 mg of dried and ground (2 mm) samples were weighed and placed into 100 ml syringes. Three blanks containing 30 mL of medium only were included in the run. Average volume of gas produced from the blanks was deducted from the volume of gas produced per sample. Gas volumes obtained at varying incubation hours were fitted to the non-linear equation model of France *et al*.[32]:

$$G = A\,(1\text{-}e^{-c\,(t\text{-}L)\text{-}d\,(\sqrt{t}\,\text{-}\sqrt{L})}) \quad (1)$$

Where G is equal to the accumulation of gas produced per unit time, A is equal to the total amount of gas produced (mL), c is equal to a fixed rate of gas production (mL per hour), d is equal to a fixed rate of gas production (mL at $h^{1/2}$), L equal to the late phase, t and t ½ time equal to half of the total gas production time is cumulative.

The amount of short chain fatty acids (SCFA) [13,33], digestibility of organic matter (DOM) [14] and ME [10,15] and microbial protein [34] were estimated using the equations below.

$$ME\ (MJ/kg\ DM) = 1.06+0.1570GP+0.0084CP+0.0220EE - 0.0081CA \quad (2)$$

$$DOM\ (DM\,\%) = 9+0.99GP+0.0595CP+0.018CA \quad (3)$$

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222GP\text{-}0.00425 \quad (4)$$

$$MP\ (g/kg\ OMD) = [19.3\ DOM\ (kg)]\times 6.25 \quad (5)$$

where: ME = Metabolizable energy (MJ/kg DM), GP gas is 24 h net gas production (mL/g DM), CP is crude protein (DM %), and EE is crude fat (DM %), CA= ash in g/100 g DM. As well as, DOM = OM digestibility (g/100 g DM), SCFA = Short chain fatty acid (mmoL), the microbial protein (MP) was calculated according to Czerkawski [34] formula that is shown in equation 5.

Methods of Data Analysis and Statistical Model

The results of the gas production test to repeated measures were analyzed using the SAS statistical software [35]. Comparing the average of the least significant difference (LS MEAN) was performed. Other data in a completely randomized design with 3 repeats and 3 treatments were evaluated and comparison of means using Duncan test when $P \leq 0.05$. Statistical model research design is as;

$$Y_{ij} = \mu + A_i + e_{ij} \quad (6)$$

where: Y_{ij} is the observation, μ is the population mean, A_i is the effects of experimental treatments and e_{ij} is the residual error.

RESULTS

The chemical composition of test feed is given in *Table 1*. Highest of CP content of 59% was obtained for a blood meal. The maximum amount of crude fat 31.3% for POM and highest ash content of 20% was achieved for FM. Highest of NDF and ADF 70.6% for CM and FM and the lowest NDF and ADF were obtained 45.7 and 33.3% for SM.

Data belong to the production of gas from fermentation of plant proteins (SM, RM and CM) and animal proteins (POM, FM and BM) with or without nano ZnO at 2, 6, 12, 24, 48 and 72 h is presented in *Table 2*.

Nutritional parameters results of gas production in *Table 3* showed that between the sources of plant protein, SCFA, ME, DOM and MP of SM was obtained by adding ZnO nanoparticles levels of 0, 30 and 60 ppm respectively (0.997, 1.034 and 0.854 mmol), (8.548, 8.810 and 7.536 MJ/kg), (56.744, 58.394 and 54.873%) and (68.427, 70.437

Table 1. *Chemical composition of some plant and animal protein sources*

Protein Sources	DM %	CP (%DM)	EE (%DM)	Ash (%DM)	NDF (%DM)	ADF (%DM)
Plant						
Soybean meal	92.4	50	1.6	6.1	45.7	33.3
Rapeseed meal	91.4	37	1.2	8	51.5	46.1
Cottonseed meal	93	24	1.4	4.7	70.6	58.4
Animal						
Poultry offal meal	94.4	55	31.3	7.3	48.9	34.8
Fish meal	93.6	50	18.1	20	61.2	40.6
Blood meal	70.6	59	1.6	5	55.3	33.4

DM = dry matter (percent), CP = crude protein (% DM), EE = crude fat (% DM), Ash = ash (% DM) NDF = Neutral detergent fiber (%), ADF = Acid detergent fiber (%)

Table 2. *Effect of Zinc Nano- oxide on the amount of gas produced (mL per 200 mg DM) at different times of some plant and animal protein sources*

Protein Sources	Levels of Nano-ZnO	Incubation Hours					
		2 h	6 h	12 h	24 h	48 h	72 h
Soybean meal	0 ppm	4.667	17.670	32.000	45.110^{ab}	55.443	58.227
	30 ppm	1.667	15.003	32.000	46.777^{a}	57.110	59.783
	60 ppm	4.667	15.337	30.667	38.663^{b}	50.660	56.667
	SEM	1.2018	1.0887	2.6105	2.2068	2.0170	1.8939
	P-value	0.2063	0.2470	0.9179	0.0870	0.1416	0.5431
Rapeseed meal	0 ppm	0.667	8.330^{a}	16.000	28.663^{ab}	39.333	41.003
	30 ppm	1.000	9.663^{a}	18.667	31.997^{a}	43.667	47.337
	60 ppm	0.000	4.997^{b}	15.333	23.997^{b}	37.000	38.337
	SEM	0.5092	0.8607	1.9626	1.667	2.0994	2.5166
	P-value	0.4219	0.0214	0.4891	0.0394	0.1540	0.1042
Cottonseed meal	0 ppm	1.000	4.500	7.000	11.333^{ab}	15.417	17.750
	30 ppm	0.667	4.333	8.000	13.000^{a}	16.250	17.750
	60 ppm	1.110	3.443	6.000	9.500^{b}	16.220	18.733
	SEM	0.2026	0.4525	0.7453	0.5357	0.8053	0.5774
	P-value	0.3402	0.2818	0.2441	0.0106	0.7217	0.4326
Poultry offal meal	0 ppm	1.333	9.670	15.667	19.997	23.997	28.337
	30 ppm	0.667	10.170	16.000	19.330	22.330	28.670
	60 ppm	0.333	8.670	14.000	17.330	20.330	24.170
	SEM	0.5773	0.5000	0.6086	1.3878	1.5031	1.7821
	P-value	0.5008	0.1780	0.1190	0.4219	0.2979	0.2187
Fish meal	0 ppm	0.000	0.663^{c}	2.333^{b}	3.830^{b}	5.500^{b}	5.670^{b}
	30 ppm	0.333	1.677^{b}	4.700^{ab}	4.577^{b}	6.200^{b}	7.670^{ab}
	60 ppm	0.667	3.830^{a}	7.000^{a}	7.330^{a}	10.500^{a}	10.670^{a}
	SEM	0.4303	0.2740	0.6995	0.6917	1.0011	1.0929
	P-value	0.5787	0.0005	0.0096	0.0262	0.0246	0.0472
Blood meal	0 ppm	0	1.000^{b}	3.500^{a}	4.500	5.083	5.750
	30 ppm	0	2.500^{a}	4.500^{a}	4.333	5.750	6.083
	60 ppm	0	1.000^{b}	2.333^{b}	3.500	5.750	6.083
	SEM	0	0.1667	0.3043	0.3043	0.5092	0.6383
	P-value	0	0.0010	0.0070	0.1190	0.5927	0.9143

** Dissimilar letters in each column represents is a significant difference (P<0.05)*

and 60.749 g/kg) that was compared with cottonseed meal and rapeseed meal the highest amount in which, due to crude protein of soybean meal. Also, SCFA, ME, DOM and MP of POM was (0.440, 0.425 and 0.380 mmol), (5.291, 5.186 and 4.872 MJ/kg) (32.201, 31.541 and 29.561%) and (38.842, 38.046 and 35.657 g/kg) between the sources of animal protein, respectively, that was the highest amount compared with FM and BM.

Results of the parameters predicted by the model France are presented in *Table 4*. As observed, the highest amount of potential gas production (A) in the case of ZnO nanoparticles at levels 0, 30 and 60 ppm was with 290.11, 297.76 and 273.26 mL per g DM for SM, respectively. Highest gas production rate constant (c) respectively with 0.180, 0.180, and 0.035 ml per hour related to the BM and lowest lag phase with values of 0, 0.427, and 0 was for CM.

DISCUSSION

Comparing fermentation gas production between plant and animal protein without adding nano-ZnO represents the total amount of gas produced of SM were highest compared with other investigated plant and animal protein sources. So, at hours of 2, 6 and 12 SM had highly produced gas compared to other sources of plant and animal proteins (P<0.01). For example, the fermented SM, after 6 h of incubation and 17.67 mL and RM and CM, respectively 8.33 and 4.50 mL of gas per 200 mg of DM (P<0.001). In 36, 48 and 72 h of incubation, although the difference in

Table 3. *Effect of Zinc Nano-oxide on parameters of nutritional some animal and plant protein sources*

Protein Sources	Levels of Nano-ZnO	DOM (%DM)	SCFA (mmol/200 g DM)	ME (MJ/kg DM)	MP (g/kg DOM)
Soybean meal	0 ppm	56.744	0.997^{ab}	8.548^{ab}	68.447^{ab}
	30 ppm	58.394	1.034^{a}	8.810^{a}	70.437^{a}
	60 ppm	54.873	0.854^{b}	7.536^{b}	60.749^{b}
	SEM	3.4059	0.0490	0.3465	2.6353
	P-value	0.7739	0.0870	0.0870	0.0870
Rapeseed meal	0 ppm	39.722^{ab}	0.632^{ab}	5.832^{ab}	47.915^{ab}
	30 ppm	43.022^{a}	0.706^{a}	6.356^{a}	51.896^{a}
	60 ppm	35.102^{b}	0.528^{b}	5.010^{b}	42.342^{b}
	SEM	1.650	0.0370	0.2617	1.9903
	P-value	0.0394	0.0394	0.0394	0.0394
Cottonseed meal	0 ppm	21.733^{ab}	0.247^{ab}	3.034^{ab}	26.215^{ab}
	30 ppm	23.383^{a}	0.284^{a}	3.295^{a}	28.205^{a}
	60 ppm	19.918^{b}	0.207^{b}	2.746^{b}	24.026^{b}
	SEM	0.5304	0.0119	0.0841	0.6398
	P-value	0.0106	0.0106	0.0106	0.0106
Poultry offal meal	0 ppm	32.201	0.440	5.291	38.842
	30 ppm	31.541	0.425	5.186	38.046
	60 ppm	29.561	0.380	4.872	35.657
	SEM	1.3739	0.0308	0.2179	1.6572
	P-value	0.4219	0.4219	0.4219	0.4219
Fish meal	0 ppm	16.127^{b}	0.081^{b}	2.317	19.453^{b}
	30 ppm	16.866^{ab}	0.097^{b}	2.435	20.344^{ab}
	60 ppm	19.262^{a}	0.158^{a}	2.739	23.235^{a}
	SEM	0.7107	0.0153	0.1313	0.8573
	P-value	0.0469	0.0262	0.1424	0.0469
Blood meal	0 ppm	17.055	0.096	2.257	20.573
	30 ppm	16.891	0.092	2.231	20.374
	60 ppm	16.065	0.073	2.010	19.379
	SEM	0.3012	0.0067	0.0478	0.3634
	P-value	0.1190	0.1190	0.1190	0.1190

DOM = digestible organic matter (%DM), SCFA = short chain fatty acids (mmol/200 gDM), ME= metabolizable energy (MJ/kg DM), MP = microbial protein (g/kg DOM), SEM = standard error of mean

the amount of gas production for each feed item is clearly visible, but the gas production in samples of fermented SM was higher compared to other plant and animal sources of protein. It seems that high level of gas production by SM due to its high levels of CP (50%) and also the ADF and NDF content was typically lower than other plant sources of protein. Also, between the sources of animal protein, gas production of BM was lower due to highly crude protein (59%) and ADF lower than other animal sources of protein. Adding ZnO nanoparticles had no effect on the *in vitro* gas production after 24 h incubation all protein sources other than FM. However, some sources tend to be significantly reduced. The volume of gas production after 24 h of incubation was used as an index of energy feed value and feed digestibility [36]. According to the observations of this study, the addition of nano-ZnO on the protein source had no effect on gas production after 24 h. However, reduction and the tendency to decrease in the volume of gas production after 24 h of incubation in other study [37] are also shown. This tends to decrease with increasing the nano level, unlike results researchers [27,28], which was up high enough so that the inhibitory effect on the activity of rumen microorganisms was shown. Between sources of animal protein, POM and BM at any of the incubation times was not affected by the addition of ZnO nano-particles, but adding ZnO nanoparticles to FM except the first 2 h of incubation at other times had significantly effect on increasing gas production. In general, the results *(Table 2)* showed that the use of ZnO nanoparticles levels of 0, 30 and 60 ppm of the gas production in 24 h different

Table 4. *Effect of Zinc Nano-oxide on gas production parameters some animal and plant protein sources by France model*

Protein Sources	Levels of Nano-ZnO	A (mL)	c (mL per h	T-Lag
Soybean meal	0 ppm	290.112	0.078	0.337^{ab}
	30 ppm	297.761	0.089	0.636^{a}
	60 ppm	273.259	0.063	0^{b}
	SEM	10.5547	0.0092	0.1276
	P-value	0.3147	0.2072	0.0215
Rapeseed meal	0 ppm	209.861	0.062	0.578^{ab}
	30 ppm	239.100	0.063	0.520^{b}
	60 ppm	199.039	0.065	0.772^{a}
	SEM	14.836	0.0070	0.0610
	P-value	0.2225	0.9682	0.0606
Cottonseed meal	0 ppm	96.592	0.037	0^{ab}
	30 ppm	89.591	0.067	0.427^{a}
	60 ppm	147.306	0.012	0^{b}
	SEM	7.0106	0.0010	0.3421
	P-value	0.0022	0.0224	0.0664
Poultry offal meal	0 ppm	131.172	0.093	0.173
	30 ppm	122.044	0.123	0.540
	60 ppm	101.769	0.148	0.637
	SEM	8.5719	0.0201	0.2509
	P-value	0.1200	0.2296	0.4382
Fish meal	0 ppm	27.496^{b}	0.105	0.710
	30 ppm	36.190^{ab}	0.074	0.246
	60 ppm	50.686^{a}	0.134	0.352
	SEM	6.0372	0.0437	0.3451
	P-value	0.0872	0.6504	0.6317
Blood meal	0 ppm	25.229	0.180	0.866
	30 ppm	29.007	0.180	0.128
	60 ppm	35.114	0.035	0.074
	SEM	3.4047	0.0665	0.2995
	P-value	0.1981	0.2813	0.1941

** Dissimilar letters in each column represents is a significant difference (P<0.05)*
A = potential gas production (mL) c = constant rate gas production (mL per hour) T-Lag = lag phase (hours)

sources of protein except POM and BM had significantly effect that this may be due to high protein content of POM and BM than other. According to these results, adding ZnO nanoparticles at levels of 60 ppm on nutritional parameters of plant protein sources, except in the case of DOM of SM that caused a significant decrease but at level of 30 ppm had no effect on nutritional parameters these sources. Between sources of animal protein, POM and BM by ZnO nanoparticles were not influenced but adding ZnO nanoparticles on DOM, SCFA and microbial protein of FM had significant difference. This showed that 30 ppm zinc element in incubation was provided rumen micro-organisms requirement in terms of the element deficiency of a nutrient needed by rumen micro-organisms. While that the level of 60 ppm, had reduced the level of these parameters or had a tendency to decline (P<0.05). Also in trials [38] of the concentration 1142 ppm of zinc element as zinc sulfate was in the diet of Jersey bull calves and observed that the concentration of volatile fatty acids, ammoniac and rumen pH did not influence by amount zinc in the diet. They reported that these values in control groups, respectively 79.08 mM, 11.10 mg/dL and 6.69 unit and treatment with zinc supplementation 81.30 mM, 10.35 mg/dL and 6.70 unit, which is aligned with the results. In another experiment, when 430 ppm of zinc element by consumption of zinc chloride, was used in the diets Aberdeen angus cows it was observed that the concentration of total volatile fatty acids, ammoniac and rumen pH were not effect by consumption of zinc [39].

According to the results, adding ZnO nanoparticles plant protein sources other than lag phase had no significant effect on *in vitro* gas production parameters, and between the sources of animal protein also, with the exception of gas production potential (A) for FM with a tendency to significant, other sources were not effect using ZnO nanoparticles. In tests conducted by Zabuli and Aliarabi [28], the amount of gas produced over 144 h of incubation in treatments containing zinc supplementation at levels 20 and 40 ppm had been used significantly different from control groups and this shows that the amount of zinc element available along with feed ingredients used in their experiments (27.50 mg per kg of DM diet), has provided the rumen microbes requirement. With regard to the increasing levels of zinc in the rumen (due to its anti-bacterial properties) can leads to reduced bacterial growth [40], It is clear that the level of 30 ppm of zinc supplements was not enough for protein sources that had an antibacterial effect on microorganisms, therefore the addition of zinc to feed on the microorganisms that are involved in the production of gas, probably had no effect , however the amount of gas production treatments containing zinc supplementation compared to control treatment had no significant increase or decrease. But with increasing level of ZnO nanoparticles from 30 to 60 ppm decrease in the production of microbial protein was found in all sources. Thus, it is observed that the results this study confirms to the findings of other researchers. As the experiments [26] levels of 0, 10 and 20 micro gram of zinc element in ml of *in vitro* in rumen fluid of Holsteins applied and its effect after 24 h of incubation on rumen fermentation was investigated and found that levels of rumen pH and ammonia levels of zinc usage was not affected. In tests conducted by Zabuli and Aliarabi [28] indicated that the use of level 20 and 40 ppm Zn from both complementary ZnO and nano-ZnO on rumen parameters both *in vitro* and *in vivo* methods had no significant effect.

Variations in the volume of *in vitro* gas production, parameters of gas production and nutritional parameters, such as ME, SCFA, DOM and MP is caused by physical and chemical properties of protein source. Accordance with results of this research, it is concluded that among the sources of plant protein, SM and in between the sources of animal protein, POM compared to other protein sources were observed suitable for ruminants due to highly in digestibility and characteristics of fermentation and nutritional value. So it seems that the potential to be included in the diet of ruminants. It was also observed that the addition of Zinc Nano-oxide at levels 0, 30 and 60 ppm had not significant difference in the amount of gas produced, gas production parameters (fermentation) and nutritional parameters different sources of protein, especially animal protein to laboratory procedures have not been effected, and therefore zinc element concentrations used in this study treatments were not high enough which could affect the fermentation process and population of microorganisms.

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Research Article

Effect of Bovine Corpus Vitreum on Full-thickness Dermal Wound Healing: An Experimental Study in Rats [1]

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Abstract

In this study, the effects of bovine corpus vitreum on wound healing were examined macroscopically and histopathologically. The study was carried out on 28 adult rats *(Rattus norvegicus)* weighing 150-300 g. Using general anesthesia, two separate wound excisions of 1 cm in diameter were formed in the right and left back halves of all rats. In the control group, the wound care was performed only by dripping serum over the wound daily. In the study group, the corpus vitreum obtained from newly cut healthy cows in the slaughterhouse was applied to the wound surface. All injuries were photographed using digital camera on days 1, 3, 5, 7, 9, 11, 13 and 15 and wound surface areas were compared. At the end of the first and second weeks, tissue samples taken from wound beds were examined histopathologically and the results were evaluated statistically. According to the wound surface area measurements and histopathologic results, corpus vitreum was found to have a positive effect on wound healing and a faster and more regular healing was achieved in the wounds compared to the control group.

Keywords: *Bovine corpus vitreum, Wound healing, Rat*

Sığır Corpus Vitreumunun Tam Katmanlı Dermal Yara İyileşmesi Üzerine Etkisi: Ratlarda Deneysel Bir Çalışma

Özet

Bu çalışmada sığır "korpus vitreum"unun yara iyileşmesi üzerine etkisinin makroskobik ve histopatolojik olarak araştırılması amaçlandı. Çalışma ortalama 150-300 g ağırlığında erişkin 28 adet sıçan *(Rattus Norvegicus)* üzerinde yürütüldü. Genel anestezi eşliğinde tüm sıçanların sol ve sağ sırt yarımında 1'er cm çapında iki ayrı eksizyon yarası oluşturuldu. Kontrol grubunda yaralara günlük olarak sadece serum damlatılarak yara bakımı gerçekleştirildi. Çalışma grubunda ise mezbahada yeni kesilen sağlıklı sığırlardan elde edilen korpus vitreum, yara yüzeyine tatbik edildi. Tüm yaralar 1., 3., 5., 7., 9., 11., 13. ve 15. günlerde dijital fotoğraf makinesi kullanılarak fotoğraflandı ve yara yüzey alanları karşılaştırıldı. 1. ve 2. haftanın sonunda yaralardan alınan doku örnekleri, histopatolojik açıdan incelendi ve sonuçlar istatistiksel olarak değerlendirildi. Yara yüzey alanı ölçümleri ve histopatolojik sonuçlara göre korpus vitreum'un yara iyileşmesine olumlu etkisinin olduğu ve uygulandığı yaralarda kontrol grubuna oranla daha hızlı ve düzenli bir iyileşmenin olduğu görüldü.

Anahtar sözcükler: *Sığır korpus vitreumu, Yara iyileşmesi, Rat*

INTRODUCTION

Healing of a wound, which occurs due to many reasons and is defined as the disruption of tissue integrity, comprises different successive processes such as bleeding and clot-scab formation period, inflammatory period, fibroplasia period, collagen period and epithelialization and maturation period [1-4]. It may be encountered various complications in the treatment process and the most important local factor that hinders these processes is infection. Different methods and materials such as placenta, collagen pets, regional antibiotic and/or epithelial pomades, antiseptics, plant extracts, insulin and local electrical stimulation have been used all along for short term completion of the healing process [1-8]. The main use of these materials is to shorten the process but the

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effect of each can be at different periods of the healing process.

Collagenase has a great importance in cell migration, is produced by epithelial cells or keratinocytes in the region at physiological pH and appropriate temperature and reaches a maximum level at day 5 [9,10]. In studies using collagen gels to accelerate healing in wound models that include all skin layers, it was observed that the wound healing process was completed in 11-15 days.

Hyaluronic Acid (HA), which is obtained directly or by isolation from cockscomb, spinal cord, skin, joint fluid and humor vitreous, is the simplest, sulphate-free member of the connective tissue protein group called glycosaminoglycan, and is actively involved in wound healing. HA, which undertakes the task of increasing the volume of vitreous fluid, also contributes to regeneration and tissue strength in the dermis and epidermis [11-13].

Vitreous is transparent, gel-like and has an extracellular matrix structure with heterogeneous viscoelastic character. Vitreous, which is around 98% water, chemically contains glycosaminoglycans, collagens, opticin, HA, chondroitin sulphate, heparan sulphate and proteoglycans. One of the important macromolecules of vitreous gel is HA and it was first isolated from cow vitreous in 1934. The other macromolecules it contains are type II (75%), hybrid type V/XI (10%), and type IX (15%) collagen fibrils. Therefore, vitreous is described as hyaluronan molecules interspersed between collagen webs [14,15].

It was reported that substances applied on wounds in order to create a humid environment better protects the vitality of the tissue by preventing wound dehydration, accelerates angiogenesis by enhancing proliferation ability of the cells and affects wound healing positively by increasing the efficiency of growth factors [16].

Bovine corpus vitreum is the ideal material containing both collagens and HA and this study focuses on its effectiveness on wound healing in experimental wound models.

MATERIAL and METHODS

The study was conducted after obtaining the approval of the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2016-046).

The animal material of the study was 28 adult rats *(Rattus norvegicus)* weighing an average of 225 g (150-300 g) obtained from the Kafkas University Experimental Animal Research Center. The rats were fed *ad-libitum* with standard rat pellet diet until the end of the study. Prior to the experiments, rats were allowed to adapt to the environment for a week. During the adaptation period and throughout the experiment, the rats were maintained at 20-22°C constant temperature and 45-55% humidity, under a 12-h light and 12-h dark cycle.

Fresh corpus vitreum was collected daily by visiting the slaughterhouse. Immediately after the cattle were slaughtered, the corpus vitreum aspirated by entering the vitreous with a 20 gauge pink cannula under aseptic conditions. The obtained liquid-gel vitreous fluid was brought to the experimental animal research and application center as soon as possible. Attention was paid to repeat the applications every 24 h. In the control group, saline was administered simultaneously with the study group instead of vitreous fluid. This practice lasted for 14 days.

Two groups were formed with 14 rats in each group. After identifying Group I as experimental group and Group II as control group, each group was categorized as I A (right), I B (left), II A (right) and II B (left). The rats were starved for 12 h prior to the operation. Anesthesia with xylazine HCl (10 mg/kg IM, Rompun, BAYER) and ketamine HCl (100 mg/kg IM, Ketasol 10%, Richter pharma) was preferred for operations and operations were performed under aseptic conditions. Following anesthesia, the right and left regions of the rats' shoulders were shaved and aseptic conditions were maintained. While creating a wound, millimeter paper was used as a guide to ensure standardization of the surface areas *(Fig. 1A)*. The wound margins were marked with previously prepared millimeter papers of 1 cm^2 in order to create wounds of equal size *(Fig. 1B)* and then a full thickness wound of 1 cm^2 in a square shape was created by the same operator by incising the skin on the right and left sides of the shoulder region of each rat in a way to include all skin thickness *(Fig. 1C)*. To the wounds formed on the right side of each of rats (Group I A, Group II A), one simple interrupted suture was applied using absorbable 3-0 polyglactin-910 (vicryl) thread *(Fig. 1D black arrow)* to prevent surface contraction. To the wounds on the left side (Group I B, Group II B), no sutures were applied to prevent contraction. Thus, the wound on the left side of each rat was evaluated as the control of the right side. Fresh vitreous fluid was applied daily to cover the entire in Group I A and Group I B and the wounds were left open *(Fig. 1D)*.

The wounds in all groups were imaged with millimeter paper using a digital camera *(Fig. 2 A)* on days 1, 3, 5, 7, 9, 11, 13 and 15 *(Fig. 3)* to measure wound surface areas. Wound surface areas were measured using Stereo Investigator version 9 (MBF Bioscience U.S) image analysis program.

At the end of the 7th day, the final images of the wound areas were taken from the first 7 subjects in each group and the wound areas were fully excised in a way to contain some intact tissue *(Fig. 2 B)*. Obtained tissues were fixed in 10% buffered formalin solution for histopathological examination. The same procedure was carried out on the 15th day in the remaining rats.

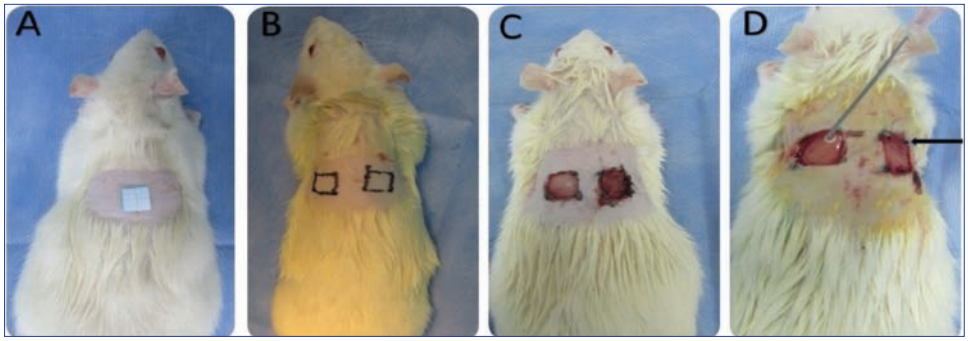
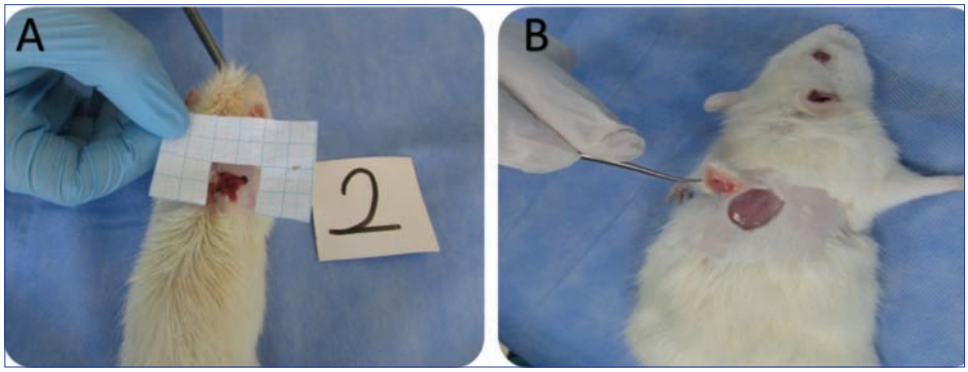


Fig 1. Creation of wound model. **A-** Use of millimeter paper as a guide, **B-** Determination of the excision line, **C-** Creation of the excision wound, **D-**Application of vitreous to the wound and suture application for prevention of contraction *(Black arrow)*

Fig 2. Daily follow-up and sampling of wounds. **A-** Imaging ImImaging of the wound with millimeter paper for surface area measurement, **B-** Incision of the wound area along with some intact tissue for histopathological examination



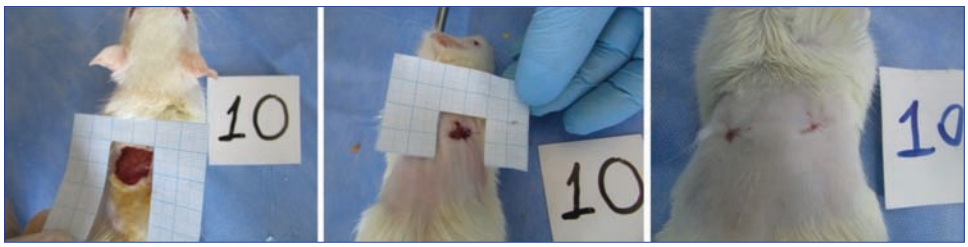


Fig 3. Images of days 0, 7, and 15 of the case number 10 of the wound area

Histopathology

On the 7th and 15th days of the study, tissue specimens fixed were routinely processed and embedded into paraffin blocks. Then sections with a thickness of 5 µ taken from the paraffin blocks were stained with hematoxylin eosin (HE) and Masson›s Trichrome stain. The sections were evaluated under a light microscope for reepithelization, presence of necrosis, neutrophil granulocyte infiltration in the areas surrounding the necrosis, granulation, neovascularization, collagen accumulation, angiogenesis, inflammatory cell volume and new connective tissue formation. The normal distrubition in the groups was examined by the Shapiro-Wilk test. The statistical comparison was made with Mann Whitney U Test.

Statistics

The data obtained from the wound surface area measurements were subjected to statistical evaluation using the Minitab 17 packet program. Each group was subjected to a test of normality. Because the groups were dependent and repeated measures were used, they were evaluated by variance analysis in repeated measures. Mean and ± standard deviation values of the groups were obtained. Values of $P<0.05$ were considered statistically significant.

RESULTS

In the daily routine maintenance and control of rats, no adverse situations such as nourishment issues or indifference to the environment were encountered. There were no deaths in the groups and no infection or similar complications were encountered in wound areas. There was no evidence of serous accumulation or an allergic condition on the wound surface in response to the vitreous used in this stage.

When the wound surface areas in Group I A and Group II A were compared, it was determined that between the 5th-13th days, the healing in Group I A was faster and the difference was statistically significant ($P<0.05$). There was also a significant difference ($P<0.05$) between Group I B and Group II B from day 5, and wound closure in Group I B was faster. A significant difference ($P<0.05$) between Group II A and II B on day 11 and a faster wound closure in Group II A was recorded also. The rate of healing in Group I B was statistically different than Group II A and II B on days

Table 1. *The values of wound surface area of groups*

Groups		1. Day (n=14)	3. Day (n=14)	5. Day (n=14)	7. Day (n=14)	9. Day (n=7)	11. Day (n=7)	13. Day (n=7)	15. Day (n=7)
Group I (cm^2)	A (right)	0.91±0.08^{a}	0.81±0.09^{ab}	0.69±0.12^{a}	0.52±0.12^{a}	0.34±0.05^{a}	0.10±0.04^{a}	0.02±0.01^{a}	0.00±0.00^{a}
	B (left)	0.92±0.02^{a}	0.77±0.07^{a}	0.63±±0.06^{a}	0.47±0.06^{a}	0.31±0.02^{a}	0.18±0.01^{a}	0.02±0.01^{a}	0.00±0.00^{a}
Group II (cm^2)	A (right)	0.93±0.01^{a}	0.85±0.03^{b}	0.77±0.03^{b}	0.70±0.03^{b}	0.60±0.04^{b}	0.38±0.05^{b}	0.28±0.03^{b}	0.04±0.06^{ab}
	B (left)	0.94±0.02^{a}	0.88±0.02^{b}	0.81±0.01^{b}	0.75±002^{b}	0.67±0.03^{b}	0.52±0.05^{c}	0.29±0.02^{b}	0.07±0.05^{b}

$^{a, b, c}$ different letters on each line show statistical differences (P<0.05)

3, 5, 7, 9, 11 and 13 *(Table 1)*. On day 15, the wounds in Group I were completely closed but the wound surfaces in Group II were not completely closed.

In all cases of Group I (Group I A and Group I B), the wound surface was almost closed on the 15th day and the new tissue formed in the healing area was similar to the intact tissue. In the control group (Group II A and II B), however, it was determined that the wound was prominently open. When the groups of rats with and without suture application were compared, there was no statistically significant difference (P>0.05) between the groups except the 11th day.

Histopathological Results

It was evaluated reepithelization, presence of necrosis, granulation, neutrophil granulocyte infiltration, collagen accumulation, inflammatory cell volume, neovascularization, angiogenesis and ulcer formation.

In the 7th day evaluation of the specimens of the groups (Group I and Group II B) that were not sutured after excision and used as control groups, it was observed that the excision surface was covered with scab, re-epithelialization had started to form at the wound edges, granulation tissue was formed beneath the scab, and a large number of new capillary formation was present. None of the specimens showed any ulceration or inflammation of the dermis. In the 7th day evaluation of the specimens of the group (Group I A) that was sutured after excision and applied corpus vitreum, it was determined that reepithelization was started and neovascularization and fibrous tissue were formed. In the specimens of the group (Group I B) that was not sutured after excision and applied corpus vitreum, it was observed that the excision surface was covered with scab. Re-epithelialization had started to form, and presence of young granulation tissue with new capillary formation was detected in the excision area. Also; in all groups, there were capillary hyperemia and occasionally hemorrhage areas in some specimens.

Masson's Trichrome staining, which was performed to detect collagen distribution in the specimens of the control group and the experimental group (Group I A-B, Group II A-B) taken on the 7th day, showed that there was irregular collagen distribution in subepidermal regions along with new connective tissue formation and neovascularization while collagen had not yet matured *(Fig. 4 A,B,C)*. There were regular bundles of mature collagen present in deep dermis.

In the 15th day evaluation of the specimens of the group (Group II B) that were not sutured after excision, the wound surface was completely closed and the epidermis was observed to be normal. There was significant fibrosis in this group but a decrease in the number of vessels. The results observed in Group I A were similar to those in Group I B. Epithelization was incomplete on in one member of this group and there was granulation tissue, congested vessels and inflammatory cell infiltration in the dermis. In addition to these results, it was observed that epidermis was thicker in the groups treated with vitreous then the other groups and papillae extended to the dermis.

Masson's Trichrome staining, which was performed to detect collagen distribution in the specimens of all groups taken on the 15th day, showed similar results and fibrosis. Collagen synthesis was present and maturing was observed in the resulting fibrous tissue *(Fig. 5 A,B,C)*.

DISCUSSION

A number of studies [1,2,5,7,8,19,20] have been carried out from the past to the present day on the completion of these cellular and biochemical phases in a shorter time by supporting the organism, thus reducing the risk of complications and achieving shorter healing. We also aimed to investigate the effect of bovine vitreous in wound healing in our study. The most basic reason for this is that biochemical structure of vitreous is formed by structural proteins such as glycosaminoglycans, collagens, opticin, fibrillin, and HA.

Cellular contraction movement, which takes place during cell proliferation stage following inflammation, one of the stages in the healing of open wounds with tissue loss, was reported to be effective 80% in closure of open wounds [6,18,19,22,24]. For acute and chronic wound models, rodents which are easy to maintain and feed, rapidly multiply and grow, and whose biological properties are well known are preferred, as in our work. However, as the link between skin and subcutaneous tissues is weak and *m. panniculus carnosus* leads to withdrawal of the wound easily, the wound is closed in a short time in these

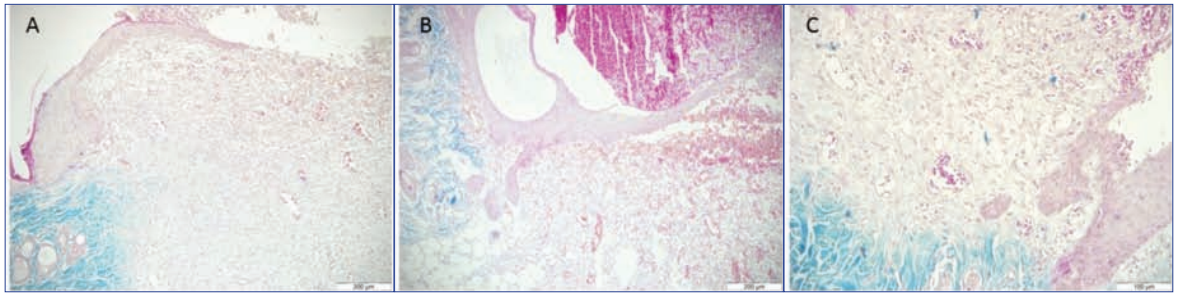


Fig 4. Histopathological view of the 7th day of the groups, Masson's Trichrom. **A-** Group II, day 7; Initiation of reepithelization, neovascularization and young granulation tissue, regular collagen fibers in bundles in the dermis, irregular collagen fibers that are not yet stainable in the newly formed fibrous tissue, Masson's Tricrom, Bar: 200 µm, **B-** Group I, day 7; Initiation of reepithelization, hyperemic vessels and bleeding, regular collagen fibers in bundles in the dermis, collagen fibers that are not yet stainable in the newly formed fibrous tissue, Masson's Tricrom, Bar: 200 µm, **C-** Group I B, day 7; Initiation of reepithelization, regular collagen fibers in the dermis, collagen fibers that are not yet stainable in the newly formed fibrous tissue, Masson's Tricrom, Bar: 200 µm

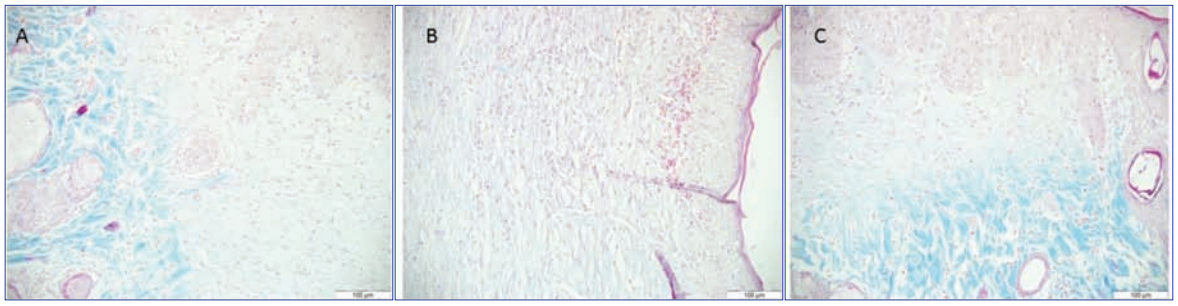


Fig 5. Histopathological view of the 15th day of the groups, Masson's Trichrom. **A-** Group II, day 15; Reepithelization, decrease in number of vessels in fibrous tissue, collagen fibers, Masson's Tricrom, Bar: 100 µm, **B-** Group I, day 15; Reepithelization, decrease in number of vessels in fibrous tissue, collagen fibers, Masson's Tricrom, Bar: 100 µm, **C-** Group I B, day 15; Reepithelialization, decrease in number of vessels in fibrous tissue, and maturation of collagen fibers, Masson's Tricrom, Bar: 100 µm

animals [1,18,23-25]. This leads to a failure to fully understand the effectiveness of the material used for healing. In this study, in order to prevent the effect of cellular movement (withdraw) in closing the wound surface, epidermis and dermal connective tissues of the square shape excisional wound were attached to muscular tissue underneath with simple interrupted sutures from four corners of the wound. So this method enabled to clearer understanding of effectiveness of corpus vitreum in wound healing.

HA is found in both the dermis and the epidermis layer, while it is present more densely in the dermis. Because of this, it is a good choice for dermal regeneration and strengthening. HA hydrogel films were reported to accelerate healing in full-thickness skin wounds [26]. It was reported that HA is effective particularly during the first stages of healing and the amount of HA in the tissue increases during the healing process [13]. Histopathologically, topical use of HA in wound healing was found to have a positive effect in cell proliferation and migration [27]. It was also reported that it increases epithelial cell migration and differentiation, decreases fibrosis and accelerates healing [13,27]. In the wound specimens examined histopathologically on the 7th and 15th days of the study, it was determined that the presence of reepithelization and young granulation tissue was more advanced than the control group. It was observed that the epidermis was thicker and sent papillar extensions towads the dermis in the groups treated with vitreous compared control groups. It was also observed that collagen synthesis was present in the fibrous tissue that formed and began to mature. These results indicate that HA and collagen in corpus vitreumu are effective at the cellular level in the process of healing and our findings are in parallel with similar studies in the literature in this respect.

In the histopathologic evaluation of Group I and Group II subjects on the 7th day, it was observed in all subjects whose wound surface was covered with scab, re-epithelialization started at wound edges, granulation tissue was formed under the scab, and a large number of new vessel formations were present. In one case of Group I A, inflammatory cell infiltration was encountered in the suture area. The presence of neutrophil granulocytes and foreign body giant cells in the suture line was interpreted as a response of the organism to the suture thread. In both groups it was observed that there was an irregular collagen distribution but the collagen had not matured. On the 15th day of the study, histopathologic evaluations revealed that the epidermis was thicker and grew papilla extensions into the dermis in the vitreous group (Group I) compared to the control group (Group II)). This result indicates that the healing of the wounds of Group I was more regular than that of Group II.

To establish a standard in the study and to ensure ease of application, vitreous fluid application was performed once a day. This biological material which was used by us for the first time for wound healing, different results may occur if it is used more than once in a day or in a longer time interval. But it is clear that there is a need for further work for this.

Although vitreous liquids are obtained from healthy cattle slaughtered in veterinary control slaughtered house, since the material in question is a material of liquid biological origin, it can be transferred mainly as prions, bacterial and viral origin. It must not be forgotten that there a risk in terms of infectious diseases. However, since the study was an experimental nature and its primary purpose was to investigate the effect of vitreous fluid on wound healing, this fact was ignored. This biological material must be converted into a commercial for use clinical trials of different species. It may be possible to use it after the product passes different safety tests. Besides none of groups had any abnormal findings that could be evaluated in this context, mainly infection.

When the data obtained from the wound surface area measurements were evaluated, corpus vitreum was found to be more effective in wound healing compared to the control group. However, no statistically significant difference was observed between Group I A and Group I B and between Group II A and Group II B. This result suggested that the sutures applied to the contour of the wound area had a stimulating effect on the area and the healing could take place at the same speed without contractions. As a result, when both macroscopic and histopathological results were evaluated together, it was found that corpus vitreum had a positive effect on wound healing. The fact that this material is readily available and has low cost suggests that it can be used in wound healing in clinical practice. However, due to the fluid nature of the corpus vitreum, it was not able to hold on to the wound surface and it has been observed to flow out in a short time due to the effect of gravity. Thus contact time between the vitreous and the wound surface is reduced. The experience we have gained from our study made us conclude that if corpus vitreum is used in conjunction with a vaseline-like carrier, the effectiveness of this material may be further increased. However, it is also clear that there is a need for further work in which other carriers are evaluated to illuminate this matter.

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Research Article

Identification of SNP Within the Sheep *RXRG* Gene and Its Relationship with Twinning Trait in Sheep

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Abstract

RXRG (Retinoic X receptor-gamma) gene was originally associated with fetal development and reproduction in human beings and animals, which was used to detect genetic variation that was associated with growth, reproduction, metabolism trait selection and breeding. The aim of this study was to detect *RXRG* gene mutation of the exon 2 and its association with twinning trait in 313 sheep and calculate litter size, genotype frequency in Chinese Merino, Hu and Kazak sheep. Polymorphism of exon 1, exon 2, exon 10 of *RXRG* gene in the study were analyzed by *PCR-SSCP,* which showed that three genotypes of P2 fragment were significantly associated with twinning traits in the analyzed population (P=0.031). Analysis of four groups of sheep showed that there was a predominate gene (B-allele) that have higher twinning rates in these individuals, which indicated that the genotypes can be chosen for twinning according to predominate genotype. In conclusion, our results strongly suggest that polymorphisms of the *RXRG* gene could be a new choice for sheep breeding and genetics through marker-assisted selection (MAS).

Keywords: *Sheep, Retinoic X receptor-gamma gene, Twinning trait, PCR-SSCP*

Koyun *RXRG* Geninde SNP İdentifikasyonu ve İkizlik İle İlişkisi

Özet

RXRG (Retinoik X reseptör-gama) geni ilk olarak hayvan ve insanlarda fetal gelişim ve üreme ile ilişkilendirildi ve büyüme, üreme, metabolizma özellik seleksiyonu ve üreme ile ilgili genetik varyasyonu belirlemek için kullanıldı. Bu çalışmanın amacı, ekzon 2 *RXRG* gen mutasyonunu ve 313 koyunda ikizlik ile ilişkisini belirleyerek Çin Merinos, Hu ve Kazak koyunlarında yavru sayısı ve genotip frekansını hesaplamaktır. Bu çalışmada *RXRG* geni exon 1, exon 2 ve exon 10 polimorfizmleri *PCR-SSCP* ile analiz edildi ve incelenen popülasyon içerisinde P2 parçacığının üç genotipinin anlamlı derecede ikizlik ile ilişkili olduğu belirlendi (P=0.031). Dört grup koyunun analizi sonucunda bir baskın genin (B-allel) bu bireylerde yüksek ikizlik oranına sahip olduğunu ve bu genotipin ikizlik için seçilebileceğini gösterdi. Sonuç olarak, elde ettiğimiz sonuçlar *RXRG* gen polimorfizminin Marker ilişkili seleksiyon (MAS) yoluyla koyun üretiminde ve genetiğinde kullanılabileceğini önermektedir.

Anahtar sözcükler: *Koyun, Retinoik X reseptör-gama geni, İkizlik, PCR-SSCP*

INTRODUCTION

Most breeds of sheep usually produce one-offspring per year, with a longer lambing interval and lower reproductive efficiency, which greatly restricting the development and production of sheep industry. Meanwhile, it is known that reproductive traits of domestic animals were controlled by a series of relevant reproductive genes that has a low heritability [1], therefore using conventional breeding methods to improve the phenotype of the reproductive efficiency in sheep that is not only time consuming, but also difficult to obtain an ideal production efficiency in the sheep industry.

Retinoic acid, as a fat-soluble small molecule, is the main

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regulator of cell differentiation and tissue morphogenesis, which plays an important role in the process of epithelial cell growth, the maintenance of visual organization, and fetal development and reproduction [2-4]. The pleiotropism of retinoic acid are mediated by retinoic acid receptor *(RAR)* and luteinized X receptor *(RXR)* that are members of the steroid/thyroid hormone receptor superfamily of nuclear receptor proteins [3,5-8]. *RAR* are ligand-controlled transcription factors that function as heterodimers with *RXR* to exert their effects by binding to specific DNA response elements, thus regulating gene expression in target cells [3,6,9-12], which were encoded by 3 different genes, including α, β and γ, and that results in the forming more types of receptors, such as *RARA*, *RARB*, *RARG* and *RXRA*, *RXRB*, *RXRG* [13]. Previous studies showed that *RXRG* gene can be expressed during pregnancy, which has a significant additive effect on litter size in the pregnant females [14]. Similarly, Huang *et al* studied found there are the relationship between genetic variation of *RXRG* and twinning trait in cow that the individuals with AB genotype of *RXRG* gene could produce more twins than those with AA genotype [15].

However, there are few reports on the relationship between *RXRG* gene and twinning trait in sheep. To explore the correlation between the genetic variation of sheep RXRG gene and its relationship with reproductive traits, in this study, genetic variation in 3 exons of *RXRG* gene (Based on the reported *RXRG* in ovis aries gene sequences) and the flanking regions were investigated in 313 sheep by using PCR-SSCP. The relationship between *RXRG* mutations and litter size in sheep was evaluated to examine *RXRG* gene as a candidate gene for sheep litter size traits. Thus, the results of the study would be beneficial to provide a theoretical basis for application on the molecular assisted breeding based on the genetic markers related to the prolificacy of sheep.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University. All samples were collected in strict accordance with the committee's guidelines.

DNA Samples and Lambing Records

Three hundred and thirteen genomic DNA samples were obtained from healthy ewes by intravenous blood collection in Xinjiang, China. All the sheep in the study aged four years and weighed 46±2.48 kg that were housed individually under the same feeding conditions including *ad libitum* access to *alfalfa* and water. All the sheep were randomly divided into four different groups were named group 1, 2, 3 and 4, respectively, among which group 1 and 2 consisted of 178 China Merino sheep with singletons and 58 China Merino sheep with twins respectively, and group 3 contained 40 Kazak sheep and group 4 includes 37 Hu sheep. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocol [16]. Besides that, all lambing records of them were obtained from the production records in the sheep farms.

Primer Design and PCR Amplification

According to the reported sheep *RXRG* gene sequences (NC_019458), three pairs of primers (P1, P2, and P3) were designed to amplify the sheep *RXRG* gene. P1 (F: 5′-CCAAAGCCTGTGGGAAACT -3′ and R: 5′- GCGGCATTATGC GTGATT -3′), P2 (F: 5′- GGGGCAACCAGATTGATTCCT -3′ and R: 5′- TCGGCAGCCTTGTCCAC -3′), P3 (F: 5′- AGCCCTGCG TTCTAT -3′ and R: 5′- AGGCGGAGGAGCAT -3′) were separately used to amplify 307 bp, 197 bp and 2204 bp PCR products for exon 1, 2 and 10 respectively. The PCR was performed in a 25 μL reaction mixture containing 0.4 μM of each primer, 200 μM dNTPs, 1×polymerase buffer (including 1.5 mM $MgCl_2$), 1 units of Taq DNA polymerase (Sangon, China) and approximately 100 ng genomic DNA as template. The cycling protocol was 5 min at 95°C followed by 35 cycles of 94°C for 30 s, X°C annealing for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min (X°C was 59, 59, 55°C for P1, P2 and P3 primers, respectively).

Single Stranded Conformation Polymorphism (SSCP)

All PCR products were subjected to SSCP analysis. Aliquots of 2 μL PCR products were mixed with 8 μL loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), denatured by heating at 98°C for 10 min and immediately placed on wet ice. Denatured samples of P1, P2 and P3 were loaded on 10% PAGE gel in 0.5×TBE buffer and constant voltage 140 V for 14-16 h after a pre-run at 220 V for 50 min. The gel was stained by a silver staining method [17].

DNA Sequencing Analysis

The 14 PCR products showed different electrophoresis patterns, which were subcloned to pMD19-T vector (Tiangen, China) and sequenced using a commercial service (Huada, Beijing, China). Nucleotide sequence alignments, translations and comparisons were carried out by using DNAMAN software, respectively.

Statistical Analysis

Genotypic and allelic frequencies and Hardy-Weinberg equilibriums were estimated. The following model was used to analyze the association between different genotypes with twinning trait: $Y_{ik} = \mu + Age_i + Marker_k + e_{ik}$, which Y_{ik} was twinning trait that was measured on each of ikth sheep, μ was the overall population mean, Age_i was type of the ith age, $Marker_k$ was the fixed effect associated with kth genotype and e_{ik} was the random error. And Chi square test for independence in polymorphic loci of the

genotypes distribution in single-twin groups [18]. Statistical analysis was carried out by SPSS for Windows 13.0.

RESULTS

SSCP and Sequence Analysis

Polymorphisms were found in the P2 fragments by SSCP analysis *(Fig. 1)*. In the P2 fragment, three unique SSCP genotypes were obtained and designated as AA, AB and BB. PCR products also indicated that there were three genotypes were sequenced in both directions in ABI PRISM 377 DNA sequencer. And subsequent sequence comparison revealed there were two SNPs of the P2 fragments in sheep in China, including g.131A > G and g.32G > A, which the former was changed to AA- and AB-genotypes and the latter was changed to BB- and AB-genotypes, however, the polymorphisms weren't found in the P1and P3 fragments by SSCP analysis.

Genetic Polymorphism of RXRG Gene in Sheep

Above all, in our study, it is worth to note that Kazak sheep is a typical single breed, Hu sheep is a typical multiparous breed, however, China merino sheep is a single and twins breed. The genotype frequency and allele frequency of *RXRG* gene in sheep were shown in *Table 1*, BB-genotype frequency of Kazak sheep in the Group 3 was higher than AB and AA, however, as well as the former one of Hu sheep in the Group 4 was also the highest but there were only two genotypes, including BB and AB. Similarly, BB-genotype frequency was higher than AA and AB of China Merino with twinning trait in the Group 2, but AB-genotype frequency was higher than AA and BB of China Merino with singletons trait in the Group 1, all of which showed B-genotype was the predominant allele in all the populations.

Genetic Characteristics of loci in Sheep Populations

Polymorphism information content, population heterozygosity, effective allele number, and Chi-square test of loci in each group were shown in *Table 2*. Polymorphism information content (PIC) of the twins and singleton populations in China Merino sheep was separately 0.328 and 0.345 that was ranged within 0.25-0.50, which indicated that the loci was moderately polymorphic in these two populations. The study results showed that the χ2 value of the loci in China Merino sheep with twins was 6.532 ($P<0.05$), and the χ2 value of the loci in China Merino sheep with singleton was 2.264 ($P>0.05$), which indicated that the mutation loci in China Merino

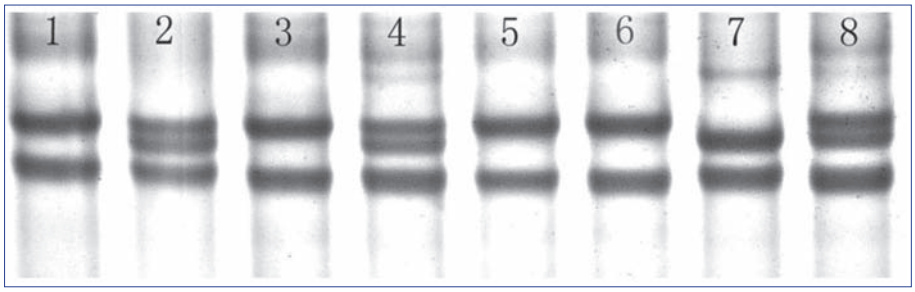

Fig 1. SSCP analysis of PCR amplification using P2
7: AA genotype 2, 4, 8: AB genotype 1, 3, 5, 6: BB genotype

Table 1. *The genotype frequency and gene frequency of RXRG gene in China Merino*

Group No.	Group	No.	RXRG2 Primer				
			Genotype Frequency			Allele Frequency	
			AA	AB	BB	A	B
1	China Merino sheep with singleton trait	178	0.084	0.494	0.422	0.331	0.669
1	China Merino sheep with twinning trait	58	0.155	0.276	0.569	0.293	0.707
2	Kazak sheep	40	0.150	0.400	0.450	0.350	0.650
3	Hu sheep	37	0.000	0.270	0.730	0.135	0.865

Table 2. *Data of Heterozygosities(H), Effective number of Alleles (Ne), Polymorphism information contents (PIC) and χ^2-test*

Group No.	Group	PIC	H	Ne	χ^2-test
1	China Merino sheep with singleton trait	0.345	0.505	1.796	2.264 ($P>0.05$)
1	China Merino sheep with twinning trait	0.328	0.724	1.708	6.532 ($P<0.05$)
2	Kazak sheep	0.336	0.643	1.747	3.436 ($P<0.05$)
3	Hu sheep	0.206	0.730	1.305	0.804 ($P<0.05$)

Table 3. *Gene frequency of RXRG in different group by χ2 -test*

Group	Group	χ2
China Merino sheep with singleton trait	China Merino sheep with twinning trait	8.986 (*P<0.05*)
China Merino sheep with singleton trait	Kazak sheep	2.138 (*P>0.05*)
China Merino sheep with singleton trait	Hu sheep	12.634 (*P<0.05*)
China Merino sheep with twinning trait	Kazak sheep	1.765 (*P>0.05*)
China Merino sheep with twinning trait	Hu sheep	6.668 (*P<0.05*)

Table 4. *Correlation analysis between different genotypes of RXRG*

Gene Locus	Breed Character	Genotype	Mean ± Std.
RXRG2	Litter size	AA	1.375±0.087[a]
		AB	1.154±0.042[b]
		BB	1.306±0.041[a]

sheep with singleton as well as the one in Kazak and Hu sheep were in the state of Hardy-Weinberg Equilibrium, but the mutation loci in China Merino sheep with twins were not in the state of Hardy-Weinberg Equilibrium, which the reason for this latter phenomenon may be that the China Merino sheep with twins were man-made intervention.

Genotype Distribution of P2 loci in RXRG in the Different Sheep Population

The genotype distribution of the polymorphic loci of the RXRG gene was analyzed in four groups using Chi-square independence test. There were significant differences between China Merino sheep with single trait and China Merino sheep with twinning trait and Hu sheep respectively ($P<0.05$), similarly, there were also significant differences between China Merino sheep with twinning trait and Hu sheep ($P<0.05$), however, there weren't significant difference between China Merino sheep with single or twinning trait and Kazak sheep *(Table 3)*.

Association Analysis Between the Polymorphism of RXRG Gene and Lambing Traits in Sheep

Through general linear correlation analysis between different *RXRG* genotypes and the litter size in China Merino sheep, compared with AB-genotype, China Merino sheep with AA-, BB-genotype were significantly higher litter size ($P<0.05$), which showed that AA- and BB-genotypes were more likely to have twins than AB-genotype. However, due to a smaller number of individuals with AA-genotype, which the difference had no statistically significant. Besides that, we also conducted a Chi-square test for independence, which there were significant differences between the individuals with singletons and twins in China Merino sheep ($\chi 2=8.986$, $P=0.011$) *(Table 4)*.

DISCUSSION

Genetic characteristics of single nucleotide mutation in this study were analyzed in the exon 2 of *RXRG* gene in sheep. There were three mutation sites of P2 site in *RXRG* that were G, T and A by comparing with the reported *Ovis aries RXRG* sequences, respectively, among which AA-genotype with an A → G mutation in 131bp and BB-genotype with a G → A mutation in 32bp were not cause amino acid changes. However, these mutations were not correlated with protein expression and therefore these mutations had not influenced gene translation [19]. Association analysis between genotype effect and twinning trait in China Merino sheep found that P2 mutation rate was higher, which showed that there were significant differences in the genotype distribution of among singleton and twins groups ($P<0.05$) and the different genotypes have a greater impact on the phenomenon of sheep twins that suggested that *RXRG* could affect the twinning trait in China Merino sheep. In this study, we selected *RXRG* gene in different sheep breeds to study the genotype frequency and gene frequency of *RXRG* gene, such as China Merino sheep, Kazak sheep, Hu sheep, which indicated that B-allele could be the predominant gene in any one of four sheep populations and it might provide a choice for selecting the sheep breeds with twinning trait.

In the previous studies, there were only few reports about the impact of *RXRG* Gene on animal reproductive traits. Messer *et al* found retinoic acid receptor, gamma *(RARG)* gene can be expressed in the critical period of pregnancy in pig, and there would be a higher of litter size in Large White pigs if *RARG* gene was expressed that the average litter size increased by 0.21 pigs [14]. Huang *et al.*[15] found that AB-genotype of *RXRG* gene in the cow would be more likely to produce twins than AA-genotype, and there were significantly different between different genotypes with singleton and twins *(P=0.0006)*. Similarly, Guo *et al.* [20] reported that the average litter size in Small Tailed Han sheep with CC-genotype of *RARG* gene were higher than CD-genetype by 0.55 *(P<0.05)*, all of which indicated that *RXRG* gene have a significant impact on the fecundity of pigs and cow. Additionally, here it is noteworthy that we also found in our experiment that the mutation site of *RXRG* gene had impact on twinning trait in sheep. There were a higher of litter size in China Merino sheep with P2 sites of AA- and BB-genotypes than AB-genotype that the average litter size increased by 0.22 and 0.15 sheep, respectively *(P<0.05)*, and importantly, there was a statistically significant significance that the individuals with BB-genotype was more likely to produce twins than

AB-genotype in sheep. In conclusion, our results strongly suggest that there were a certain correlation between polymorphisms of the *RXRG* gene and twinning trait that could be a new choice for sheep breeding and genetics through *MAS*.

Competing Interests

There are no potential conflicts of interest.

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Research Article

Contributions of Foreign Scientists to the Turkish Veterinary Medicine and Animal Husbandry in the Early Republican Period (1923-1933) [1]

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Abstract

In early republican period through Ghazi Mustafa Kemal Atatürk's aim to raise the nation to the level of contemporary civilizations, a large number of acknowledged specialists were invited from numerous countries and these specialists provided remarkable contributions to the fields of veterinary medicine and animal husbandry in Turkey. These specialists performed visits to livestock establishments and laboratories across the country and carried out research and inspections and then submitted reports to the Ministry of Agriculture. The research on the history of veterinary medicine shows that there has been no comprehensive study that collectively evaluates all of the specialists invited from foreign countries for the improvement of the fields of veterinary medicine and animal husbandry. With this study, which was aimed to investigate the specialists that arrived in Turkey in the early Republican period (1923-1933), 14 brief data relating the specialists were prepared by using archival documents, Ministry of Agriculture Specialists Reports and other sources and 11 photos have been reached. It can be said that the reports of foreign specialists coming to Turkey are presented for the development of animal husbandry and veterinary medicine and that the various opinions and suggestions in the reports can be regarded as important contributions to the progress of the country.

Keywords: *Foreign scientists, History, Republic, Turkey, Veterinary medicine*

Cumhuriyet'in Erken Dönemlerinde (1923-1933) Yabancı Bilim Adamlarının Türk Veteriner Hekimliği ve Hayvancılığına Katkıları

Özet

Cumhuriyet'in erken dönemlerinde Gazi Mustafa Kemal Atatürk'ün önderliğinde ülkenin çağdaş ülkeler seviyesine çıkarılabilmesi amacıyla birçok ülkeden alanında uzman bilim adamlarının getirilmesi, veteriner hekimliği ve ülke hayvancılığına hatırı sayılır katkılar sunmuştur. Bu uzmanlar çeşitli illerde hayvancılık işletmelerini-kurumlarını ve laboratuvarları ziyaret ederek araştırma ve incelemelerde bulunmuş, birçoğu görüş ve önerilerini raporlar halinde Ziraat Vekâletine sunmuştur. Veteriner hekimliği tarihi araştırmaları kapsamında erken cumhuriyet döneminde (1923-1933), ülke hayvancılığına ve veteriner hekimliğe katkı sağlamak üzere yurt dışından ülkeye gelen bilim adamlarını ele alan bütünsel bir araştırma bulunmamaktadır. Bu dönemde, Türkiye'ye gelerek hizmetlerde bulunan bilim adamlarını saptamak ve katkılarını belirlemek amacıyla gerçekleştirilen çalışmada, arşiv belgeleri, Ziraat Vekâleti Mütehassıs Raporları ve diğer basılı kaynaklardan elde edilen veriler biraraya getirilmiştir. Bu uzmanlardan 14'üne ait bilgilere ve 11 uzmana ait fotoğrafa ulaşılmıştır. Türkiye'ye gelen yabancı uzmanların çalışmaları sonucunda hazırlanan raporların, ülke hayvancılığı ve veteriner hekimliğin gelişmesi amacıyla sunulduğu, raporlarda yer alan çeşitli görüş ve önerilerin ülkenin ilerleyebilmesi adına önemli birer katkı olarak değerlendirilebileceği ileri sürülebilir.

Anahtar sözcükler: *Cumhuriyet, Tarih, Türkiye, Veteriner hekimliği, Yabancı bilim adamları*

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INTRODUCTION

There have been lots of contributions to the Turkish veterinary medicine and animal husbandry by the scientist who came from different of countries since the variety of relations have been established during the 19th and 20th centuries [1,2]. The scientific aspects have been improved with the Turkish Republic which was founded after the World War I [3]. During the İzmir Economic Congress held in 1923, topics: increase on the number of animals, animal breeding and the preventing of diseases have been highlighted while the Lausanne Peace Treaty meeting was continuing which caused the Turkish War of Independence to end. *"Five Year Program of Veterinary Services"* prepared by the General Manager of Veterinary Services Mr. Ali Rıza (Erem) have been applied from 1924-1925 period; topics animal breeding, preventing of diseases, training of veterinarians took place [4]. In republican period -like many other fields- lots of well-known scientists have been invited to the Turkey for the improvement of veterinary medicine and animal husbandry. Accompanied by translators[1,2], these scientists have contributed to the Turkish veterinary medicine about educational issues, epidemics, animal health, breeding and husbandry.

Within the frame of modernization on higher education; German professors are assigned for reaching to the level of contemporary countries, and made various publications in Higher Institute of Agriculture (HIA) established in 1933 [1,2,5-7]. However, it is determined that there is no holistic research has been carried out about other foreign specialists who came from different countries. This study aimed to determine these scientists, their contributions and services to the scientific advancement of veterinary medicine in Turkey between the period of 1923-1933.

MATERIAL and METHODS

Main material of the study was the documents of Department of Prime Ministry Republican Archive and 240 pages of Ottoman scripted *"Ministry of Agriculture Specialists Reports"* (Ziraat Vekâleti Mütehassıs Raporları) attained from bibliopoles. The study has been limited only with the foreign specialists assigned by Ministry of Agriculture between the years of 1923-1933. Data for accessible 14 specialists have been evaluated chronologically according to the arrival time; the tags and the needed explanations of the original documents have been shown on the footnotes.

RESULTS

1. Prof. Dr. Paul Forgeot

Bacteriologist of Paris Pasteur Institute, Prof. Dr. Paul Forgeot *(Fig. 1-a)* first arrived to the İstanbul in 1914 as the manager of *"Veterinary Bacteriology Institution";* he had to go back his country because of the World War I. After the proclamation of Republic, he again came back from Paris to the Turkey by the invitation of Government and ordinarily served as a professor at the same place in Pendik between 1925-1929. At the same time, he regularly gave conferences and consultancies and took place in scientific research.[3, 4, 5, 6, 7] Professor Forgeot reported[8] his experiences and opinions about *pasteurellosis* and *salmonellosis* to the Ministry of Agriculture in 1927 and stated that the vaccine or serum was not prepared for goat pasteurella but chicken cholera for avian pasteurella, and pig peripheral blood serum and vaccination for pig pasteurellosis have been prepared. According to the Forgeot, vaccines can not be used for infectious pneumonia in goat because the vaccines of Russia have been tested on different animals. According to the Maurice Nicole and Mr. Refik, in this disease, microorganism called *"microbe dömorti (de morte)"* and other microorganisms could be important and infection should be evaluated again. Experiments should be carried out on laboratory animals instead of goats because the factor could cause death by changing its forms. Forgeot also gave detailed information and actions to be taken (even about staff procurement and adequate salaries) about infection.

2. Prof. Dr. Jan Rostafiński

Prof. Dr. Rostafiński *(Fig. 1-b)* was invited by the Turkish Republic from Warsaw in 1924 and made researches on Western Anatolian animals.[9] According to the report[10] of Rostafiński presented to the Ministry of Agriculture in 1927: He came to the Turkey by the invitation of Minister of Agriculture Mr. Şükrü (Kaya) in 1924. Accompanied with Şefik (Kolaylı) (Ministry of Agriculture Bacteriologist) and Mr. Yaşar (İzmir Agricultural Director) he visited Eskişehir (Çifteler Studfarm), Bursa (Karacabey Studfarm), İzmir (Agricultural School, Buca, Selçuk), Aydın (Söke), Denizli (Ömerli Stallion Storage), Afyonkarahisar (Dinar ve Sandıklı) and Ankara in order examine West Anatolian animals. He presented his evaluations about horses, cattle, sheep and promised an extra report. Most part of the report was about the situation of Karacabey Farm. Prof. Dr. Rostafiński stated that because of the lack of investigation; no evaluation has been made. He also thanked the Counsellor Mr. Ali Rıza for his benefactor efforts.

[1] BDA-CAB, 30. 18. 1. 2/12. 47. 19 (05.07.1930).
[2] BDA-CAB, 30. 18. 1. 2/13. 51. 16 (24.07.1930).
[3] BDA-CAB, 30. 18. 1. 1/18. 23. 10 (28.03.1926).
[4] BDA-CAB, 30. 18. 1. 1/25. 50. 14 (21.09.1927).
[5] Anonymous: Profesör Dr. Forgeot. *Türk Baytarlar Birliği Dergisi*, (1): 167, 1936.
[6] Anonymous: Bilim dünyasında bir kayıp. *Türk Vet Hekim Der Derg*, 28 (136-137): 29, 1958.
[7] Batu A: Pendik Veteriner Kontrol ve Araştırma Enstitüsü (1901-1982). Pendik Veteriner Mikrobiyoloji Enstitüsü Yayın No: 6, İstanbul, 1982.
[8] Dr. Forgeot's report. Türkiye Cumhuriyeti Ziraat Vekâleti, Mütehassıs Raporları Baytar Kısmı, İstanbul Hamid Matbaası, 1927, p.109-111.
[9] Bekman M: Veteriner Tarihi. Ankara Basım ve Cildevi, Ankara, 1940.
[10] Dr. Rostafiński's Report. Türkiye Cumhuriyeti Ziraat Vekâleti, Mütehassıs Raporları Baytar Kısmı, İstanbul Hamid Matbaası, 1927, p.1-31.

Fig 1. Some foreign scientists. a: Prof. Dr. Paul Forgeot, b: Prof. Dr. Jan Rostafiński, c: Dr. M. Çiki Ferans, d: Dr. Heurn, e: Prof. Dr. Hans Ganslmayer, f: Dr. Heufmeister, g: Dr. Felix Lestoquard, h: Dr. Franz Gerlach, i: Prof. Dr. Oscar Wellemann, j: Dr. Hackel, k: Dr. Tadeusz Vetûlani [9,10,11,12]

3. Dr. M. Çiki Ferans

Dr. M. Çiki Ferans *(Fig. 1-c)* has been invited from Hungary for the coordination of Karacabey Studfarm Horse Breeding Brach by Ministry of Agriculture and has been positioned as the Manager of Horse Breeding branch between 1925-1939.[11, 12] In his report[13] about the improvement of Turkish horses in 1931, he highlighted the need for a well coordinated horse breeding and also stated the importance of native horse breeding in consideration of the geographical position of the country. He also marked that hybridisation of Arabian Horses which seen to be similar to the native races, with local horses can give better results. Dr. Çiki Ferans has also stated his evaluations about pregnancy state of the studfarm horses on the Journal of Turkish Veterinary Association.[14]

4. Dr. Heurn

Zootecnist Dr. Heurn *(Fig. 1-d)* has come to the Turkey to perform scientific expedition by the invitation of Turkish Republic Government in 1926.[9] In his report[15] written by the order of Ministry of Agriculture; he stated his observations about Çifteler Studfarm and Seydi Plain/ Mahmudiye/Eskişehir, Karacabey/Bursa, Adana, Gaziantep, Maraş, Karaköprü-Urfa and Diyarbakır respectively during his travels with Mr. İhsan Abidin (Akıncı). Besides the positive findings, he also mentioned deficiencies related for the pasturage improvement and animal breeding. He also mentioned about lack of effort in animal breeding, fall through in terms of expectations of production, need for a local and high quality aimed policy, advantageous price of local animals instead of importing, etc. Dr. Heurn showed the public's interest for horse-racing and draw attention to the lack of equipment in Karacabey, importance of cooperation between military and civil veterinarians and their family relations, role of official veterinarians/ agriculturists and task of government for providing qualified animals. Dr. Heurn also presented a statement in Ankara about the Report of Horse Congress held under the presidency of Acting Minister of Agriculture Mr. Cemil and indicated the risk of animal importing in terms of the future of native animal breeding.

5. Prof. Dr. Michailov

The Government of Turkish Republic made a decision about bringing Russian Scientist Ilya Ivanovich Ivanov or one of his suggested colleague to the Turkey in 1926[16]; after the invitation, Russian Scientist Prof. Dr. Michailov came to the Turkey and started to research about artificial insemination with veterinarians Nazım (Uygur) and Tevfik (Bulak) in Karacabey Studfarm[17]. In his report[18] presented to the Ministry of Agriculture, he stated his mission as to apply his experiences about artificial insemination to the animals of Karacabey Studfarm. He remarked that 24

[11] BDA-CAB, 30. 18. 1. 1/27. 81. 20 (19.02.1928).
[12] BDA-CAB, 30. 18. 1. 2/34. 11. 6 (25.02.1933).
[13] Çiki: Türkiye atlarının ıslahı hakkında rapor, Türk Baytarlar Cemiyeti Mecmuası, Gayri Mevkut, (6): 131-133 and (7): 103-107, 1931.
[14] Çiki: Karacabey Harasında kısraklarda gebelik nispeti ve müddeti üzerindeki müşahedeler, Türk Baytarlar Cemiyeti Mecmuası, Gayri Mevkut, (3):41-46, 1930.
[15] Dr. Heurn's Report. Türkiye Cumhuriyeti Ziraat Vekâleti, Mütehassıs Raporları Baytar Kısmı, İstanbul Hamid Matbaası, 1927, p.85-87 and p.96-108.
[16] BDA-CAB, 30. 18. 1. 1/18. 20. 9 (16.03.1926).
[17] Atabek S: Tiftik keçisi sürüsü üzerinde yapılan sun'i tohumlama. *Türk Baytarlar Birliği Dergisi,* (2): 275-285, 1936.
[18] Dr. Michailov's Report. Türkiye Cumhuriyeti Ziraat Vekâleti, Mütehassıs Raporları Baytar Kısmı, İstanbul Hamid Matbaası, 1927, p.87-95.

mares (three of them owned by the villagers) and two cows have been given to him and 17 mares have been artificially inseminated by five veterinarians. In addition of the report specifications: *"name of the mare", "status of uterus and vagina", "name of the stallion", "time of artificial insemination", "amount of sperma", "qualities of sperma", "amount of sperma given to the mare for the first insemination" "amount of sperma given to the mare for the second insemination", "third insemination and considerations"* have been presented respectively.

6. Prof. Dr. Hans Ganslmayer

Viennese Bacteriologist Prof. Dr. Hans Ganslymayer *(Fig. 1-e)* has served as a specialist in Ankara Etlik Military Veterinary Bacteriology and Serology Institute between 1926-1928. In his report[19] to Ghazi Mustafa Kemal Pasha in 1928, Ganslmayer reported his views on the situation of military and civilian veterinarians and stated that the military veterinary profession is in European standards in general but that the plague disease seen in bovine animals is a big problem. While the military can not make a priority against this disease, the Turkish Government emphasized that the concept of the seriousness of the work was the result of the production of the necessary serum, but the amount of production was not sufficient. Dr. Ganslmayer stressed that the civil veterinary medicine is also very responsible and civil veterinarians should be adjusted to the new needs of modern Turkey. The Government of İsmet Pasha developed the animal breeding by taking this issue seriously. Dr. Ganslmayer stated that the number of specialists in the field of veterinary medicine is insufficient and suggested that free intervention to animal diseases, establishment of institutions providing drugs and serum against the plague and cooperation with an international organization should be regulated.

7. Dr. Heufmeister

Epidemiology Specialist Heufmeister *(Fig. 1-f)* served at the Ministry of Agriculture between 1926 and 1928[9]. Dr. Heufmeister stated in his travel report in 1926 that despite the lack of personnel in the İstanbul Veterinary Directorate, the work intensity was well managed and that the needs for service were met on time. He stated that there is a lack of personnel in the quarantine stations due to the struggle with cattle plague, the fact that the legislation is sufficient for quarantine but additional precautions such as baths to fight acarids in dogs and cats places where imported animals are to be kept should be present. He also mentioned that the slaughterhouses are in very good condition but detailed instructions on meat inspection are necessary. Dr. Heufmeister, regarding the Higher Veterinary School; stated that despite the impossibilities (substructure, instrument etc.) and distance from the city, education and animal health care were carried out very well, that the country was in dire need of well-trained veterinarians, that the salaries of teachers had to be improved, and international research was important for enhancing experiences. In his assessment about the Pendik Veterinary Bacteriology Institute, he has stated that the Institute has shown that it is competent with domestic production and that the investments to be made in the Institution will provide a great contribution to the economy of the country for future periods. In addition to this, it has been suggested that the cattle plague serum should be produced with the construction of local barracks in places where the disease can be seen; instead of this institution in Pendik. Dr. Heufmeister, in his evaluations in Bursa (Mudanya Quarantine, Mudanya Veterinary Directorate, Bursa Veterinary Directorate, Karacabey, Mustafakemal-paşa-Kirmasti), Kütahya, Balıkesir, Manisa and İzmir emphasized that it is not possible to diagnose diseases such as piroplasmosis and barbitone which can not be detected by inspection because of the lack of microscopy and the importance of establishing a library to develop professional knowledge and skills to follow developments. Dr. Heufmeister has examined piroplasmosis, horse boil disease, mange, glanders, distomatosis, *streptococcus agalaxie* in goat, dog and cat, foot rot in sheep and the camel smallpox and stated that there are number of important responsibilities for veterinarians in terms of struggling these, in a time that the number of veterinarians are already insufficient. Dr. Heufmeister stated that the veterinarians in the field of fight against pandemic animal diseases must have sufficient equipment (tool bag, micro-scope etc.) and professional documents, participate in applications abroad to improve their professional know-ledge and skills, make food and milk examinations in cities, be provided with appropriate salary. He also notified that these deficiencies are the reason for the lack of number of active veterinarians. He pointed out the importance of providing enough material to fight the diseases, giving notice to disinfection, transporting the samples to Ankara in case of disease, quarantine points and a simple laboratories in slaughterhouses at least. He also mentioned about the diseases: cattle plague, glanders, mange, lenfangitis epizootica and the importance of border control.

8. Prof. Dr. Oldenburg

An Agricultural Expert Commission from Germany was invited by the Government of the Republic of Turkey in 1927 to examine agriculturel subjects and prepare a report on higher education of agriculture. This delegation, composed of 11 people under the presidency of Prof. Dr. Oldenburg[20], came to Turkey and performed examinations in 1928. Prof. Dr. Oldenburg presented a report[21] about examinations as the chief of Commission and Government

[19] BDA-CAB, 30. 10. 0. 0/186. 282. 16 (12. 1928).

[20] BDA-CAB, 30. 18. 1. 1/28. 19. 6 (01.04.1928).

[21] Oldenburg Report: German Agriculture Specialists team's report about the studies between 1 April – 30 September 1928, *Türkiye'de Altı Aylık Kuruluş Çalışmaları*, Has been duplicated by Dr. Oldenburg; did not published (Widmann H: Atatürk ve Üniversite Reformu. Kabalcı Yayınevi:159. p.66, 2000).

principal consultant. In this report, proposal that an institution should be opened especially for contemporary higher education and research has been suggested. This delegation, called the Oldenburg Delegation, took part in the preparations for the establishment of the Ankara HIA and also visited the Etlik Bacteriology Laboratory [2,8].

9. Veberman

It has been determined that the Estonian Specialist Veberman came to Turkey in 1930 with the invitation of the government to reform and improve the fishery.[22]

10. Jneral Hayni

Jneral Hayni, Director of the Meyzo Heygeş Studfarm[23] in Hungary, which was one of the most important studfarms of the world, came in 1930 with the invitation of the Government of the Republic of Turkey and took part in the establishment and administration of studfarms and stallion stores.[24]

11. Dr. Felix Lestoquard

One of the chiefs of Algeria Pasteur Institute, Bacteriologist Felix Lestoquard *(Fig. 1-g)* was commissioned by the Government of the Republic of Turkey for three months in order to investigate suspicious diseases of Merinos sheeps in Karacabey Studfarm in 1931.[25] He recognized internationally for his research on piroplasmosis, and has extensively investigations on various animal diseases, including *"sheep malignant anemia, sensitivity of buffalos to the cattle piroplasmosis and pernicious anemia of sheep and goats"*. Dr. Lestoquard had general determinations about laboratory conditions of studfarms, sheep's presence and viruses in his report.[26] In the first part of the report, presence of piroplasmosis in imported Merinos's, causes of pernicious anemia in sheep and goats, studies on their diagnosis and treatment and suggestions took place and in the second chapter, necessary precautions against diseases have been explained. During his time in Turkey, Lestoquard also gave a lecture in İstanbul Veterinary School besides these examinations[27].

12. Prof. Dr. Franz Gerlach

Dr. Franz Gerlach *(Fig. 1-h)* whom Professor of Mödling Bacteriology Institute, Vienna first came to Turkey in 1932 and studied on the scientific studies of Bacteriological Institutes.[28] Prof. Dr. Gerlach published his research on epidemics in Turkey in 1933 and then came to Turkey for the second time to serve as a specialist in the Pendik Bacteriology Institute between 1951 and 1953.[7,9,29]

13. Prof. Dr. Oscar Wellemann

Rector of Zootechny of Budapest Veterinary High School, Dr. Oscar Wellemann *(Fig. 1-i)* was invited to Turkey in 1932[30] and 1933[31] by the Government of the Republic of Turkey to investigate and report on the activities of the country animals, the measures to be taken, examine the studfarms, stallion stores, sheep pens and cowships. Dr. Wellemann made researches about horse breeding in Turkey and presented his findings to the Ministry of Agriculture in two reports[32], which were published in 1938. In his first report, written in 1932, he informed to the authorities about seven weeks of his visits and his observations on features and zootechnics of state studfarms and stallion stores. The first part of the report gives general information; it has been mentioned that animal husbandry has an important place in the economy of the country, improvement of pasture and meadows, increase of nutrient content of animal feeds and widespread cultivation of agriculture among large populace. He also emphasized that Turkey has well-adapted precious animals and therefore specialists should be trained and animals should be improved under regular selections and good care. Information has been given about animal existence, species, qualities and breeding with the topics of "*Horse Breeding*", "*Cattle Breeding*" and "*Sheep Breeding*". Dr. Welleman stated his observations concerning Karacabey, Çifteler, Sultansuyu Studfarms, Uzunyayla, Mercimek, İnanlı Stallion Stores and Cowship, Edirne Stallion Store and Kırklareli Stallion Store. In the second report of Dr. Wellemann to the Minister of Agriculture in 1933, he expressed his appreciation for consideration of his suggestions and the developments in Karacabey Studfarm. He also expressed negative and positive assessments about stallion stores in İstanbul, Bursa, Erzurum, Kars, Karaköse, Uzunyayla, Ankara and emphasized the importance of planning and programming.

14. Karl Kahl

German Specialist Karl Kahl[33] came to Turkey in 1933 with the invitation of the Government and worked on the construction and repair of sensitive glass materials at the Ankara HIA.

[22] BDA-CAB, 30. 18. 1. 2/12. 44. 13 (21.06.1930).
[23] BDA-CAB, 30. 10. 0. 0/185. 278. 18 (20.12.1931).
[24] BDA-CAB, 30. 18. 1. 2/13. 53. 17 (29.07.1930). In this archive document, Jneral Hayni has not been titled as specialist, however, the statements *"… who is capable of management of studfarms and stallion stores"* and *"expenses from the class of specialists…"*is reviewed as that Jneral Hayni is a specialist. Besides that, because of Jneral Hayni is mentioned as specialist in Footnote: 21, he has been included to the study.
[25] BDA-CAB, 30. 18. 1. 2/21. 46. 10 (24.06.1931).
[26] Cezair Pastör Enstitüsü Lâboratuar Şefi Lestokar'ın Karacabey Harasındaki Hastalıklar Hakkında Tetkik Raporu. Türk Baytarlar Cemiyeti Mecmuası, Gayri Mevkut, (12): 2-31, 1933.
[27] Golem SB: Felix Lestoquard (1797-1940).*Türk Veterinerler Cemiyeti Dergisi,* 13 (4): 93-95, 1945.
[28] BDA-CAB, 30. 18. 1. 2/27. 23. 7 (04.04.1932).
[29] Kolaylı Ş: Bakteriyoloğ Dr. Frantz Gerlach'ın memleketimizden ayrılması münasebetiyle. *Türk Vet Hekim Der Derg,* 23 (78-79): 716-719, 1953.
[30] BDA-CAB, 30. 18. 1. 2/28. 35. 1 (01.05.1932).
[31] BDA-CAB, 30. 18. 1. 2/38. 56. 8 (05.08.1933).
[32] Dr. Oscar Wellemann, Ziraat Vekâletine Verilen Raporlar, Hüsnütabiat Matbaası, İstanbul, 1938, 52p.
[33] BDA-CAB, 30. 18. 1. 2/36. 32. 2 (26.04.1933).

DISCUSSION

It has not been possible to reach all the names and numbers of foreign specialists who contributed to the veterinary profession and animal husbandry of Turkey. Kolaylı [13] evaluated that: *"The number of the European* specialists *who were brought after the Republic period is only known by God."* This assessment also explains the greatness of the number of specialists. Dinçer [2] also reported that 40 scientists have been invited until 1943; and Batu [14] stated that almost 30 foreign specialists invited only for the Pendik Veterinary Control and Research Institute between 1965 and 1982. Considering the increasing number of institutions related to veterinary medicine and animal husbandry, it may be considered that numerous of foreign specialists came to Turkey may have taken part in various institutions and organizations belonging to veterinary medicine and these statements also reveal the limitations of the study.

In some of the sources identified by the study [9,15,16-18] it was reported that Dr. Hackel *(Fig. 1-j)*, Prof. Dr. Tadeusz Vetûlani *(Fig. 1-k)* and Prof. Dr. M. Adamezki came to Turkey in the same period and for similar purposes. Dr. Hackel was appointed as a surgical specialist at the Military Veterinary Practice School served there from 1926 to 1930 while he was the head assistant of the Vienna Veterinary School Surgery Clinic [9,15]. Prof. Dr. Tadeusz Vetûlani, Director of Etienne Bathory University, Animal Breeding Institute, came to Turkey in 1929 and 1934 and prepared reports on the origins and breeding of domestic animal races, especially Arabian horse, sheep and goat. Dr. Vetûlani also visited Ankara HIA [16,17]. Upon the demand of veterinary and agriculture schools and the invitation of the Minister of Agriculture in 1933, Zootechnist Professor Dr. M. Adamezki came to the Turkey from Vienna and gave a lecture to the students of the Higher Veterinary School in Darülfünun. Dr. M. Adamezki gave some informations about the breeding and origins of animal races, especially sheep and goats. He has also visited the Karacabey Studfarm [18].

Bekman [9] showed the given importance to the animal breeding and veterinary medicine on early republic period in his statement: *"One of the aims of the newly established state was populism. Government aimed every business, every enterprise to be very productive for the benefit of public, villagers and breeders; in this way, veterinary works was also aimed to be very effective. To that end, foreign professors and high scholars have been invited from abroad to investigate the hometowns and show measures about animal welfare and treatment; receive reports on the necessities of animal diseases in the country, and on the other hand, commissions composed of important persons were working for veterinary services to be based on productive principles and walk through error-free roads."*

Yaşar [19] reported that internationally recognized foreign scientists in their field, among them Prof. Dr. Michailov, Prof. Dr. Welleman, Dr. Çiki and also one of them Ilya Ivanovich Ivanov were assigned to studfarms since 1924. Foote [20] reported that Russian Scientist Ilya Ivanovich Ivanov is known as the first person to perform modern artificial insemination technics in farm animals in 1922. In the study, invitation of Ilya Ivanovich Ivanov, and Prof. Dr. Ganslmayer's presentation of a report to Ghazi Mustafa Kemal Pasha *"in person"* in 1928 can be regarded as an indication of the importance of the newly established Republic to the science and scientists. As Yaşar [19] points out, it can be said that since the first years of Republic, has been shown an effort in order to provide a modern sence of service to studfarms for reach western standarts.

Bekman [9] reported that scientists gave a lot of detailed reports on their work, that consideration of specialist reports and as a result of the activities of the country's veterinarians, an increase in veterinary work began to be felt day after day. According to Dinçer [21] and Özkul [22] Prof. Dr. Michailov's initiative in the artificial insemination research studies and applications in Turkey since 1926 was the second application after the Soviet Union in the world. With the date Ivanov or a specialist to be appointed is invited, Prof. Dr. Michailov's arrival date to the country is the same (1926), therefore it can be argued that Michailov may be the specialist recommended by Ivanov. Osmanağaoğlu and Melikoğlu [23] stated that with the invitations of specialists Prof. Dr. Oscar Wellemann and Dr. Çiki; they have contributed for the improvement of zootechny and breeding institutions; Menteş Gürler et al.[11] stated Prof. Dr. Rostafinski's contributions to the animal husbandry with his studies. It is possible to say that these findings support the data of the reports in the study and that the arrival of scientists to our country is a great gain for the reform movements. In fact, the Studfarm and Breeding Institutions established after the proclamation of the Republic have achieved considerable success in the following years, and foreign scholars visited these institutions reported such successes in their reports [24]. It is understood that with the cooperation of foreign specialists and Turkish Veterinarians, the knowledge of western countries is transferred to Turkey, and the applications made with new techniques and methods have increased the quality of veterinary medicine and animal husbandry. In the following years, Kolaylı [13] reported that *"reports given by the specialists brought after the Republican era have been taken to a corner. There is no doubt about that"*. It may be though that there was a decline in veterinary medicine and animal husbandry in that period, but it can only be determined in future studies that there has been a regression in the following periods.

Among the invited specialists Rostafinski, Çiki and Welleman emphasized the importance of domestic breeding and domestic investment and in this way, it is foreseen that domestic crops will increase in yields and therefore

domestic production. Today, animal importation is not recommended in terms of sustainability of production as well as of protection of national interests [25], and the Ministry of Food, Agriculture and Livestock of Turkey forbids the import of animals from various countries due to diseases [26]. It can be said foreign scientists' repudiate of import policiesis meaningful and important to think of when the risk of emerging new diseases due to the risks of imported animals in terms of resistance and climatic adaptation is considered.

It can be said that the reports of foreign specialists coming to Turkey are presented for the development of animal husbandry and veterinary medicine and that the various opinions and suggestions in the reports can be regarded as important contributions to the progress of the country.

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Research Article

Melissopalynological Analysis for Geographical Marking of Kars Honey [1]

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Abstract

In this research, the melissopalynological analysis of honey samples collected from Kars city located in the East Anatolian Region of Turkey was conducted for geographical marking. Within this context, melissopalynological analyses of 100 honey samples determined by sampling method were collected from eight districts of Kars in Eastern Anatolia Region of Turkey were done, to determine the nectarous source plants of Kars honey. As a result of melisopalynological analyses carried out in 100 honey samples; pollens of the taxa belonging to Apiaceae, Asteraceae, Berberidaceae, Betulaceae, Brassicaceae, Boraginaceae, Campanulaceae, Caryophyllaceae, Chenopodiaceae, Cistaceae, Cyperaceae, Dipsacaceae, Ericaceae, Fabaceae, Iridaceae, Lamiaceae, Liliaceae, Malvaceae, Onagraceae, Papaveraceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rhamnaceae, Rosaceae, Rubiaceae, Rutaceae, Salicaceae and Scrophulariaceae families were detected at different rates. Almost in all of the honey samples, *Lotus corniculatus* (in 99 samples), *Onobrychis radiata* (in 99 samples), *Trifolium nigrescens* (in 88 samples) from Fabaceae family and pollens of *Echium vulgaris* (81 samples) and *Myosotis lithoospermifolia* (15 samples) taxa from the Boraginaceae family, were found in honey samples. *Onobrychis radiata* pollen was the most intensely observed one among these samples (in dominant, secondary, minor, trace amounts). The total number of pollens (TPN-10) in 10 grams of honey were also detected during the melissopalynological analyses. TPN-10 values minimum: 226, maximum: 481157 and mean: 31678 were detected and the pollen abundance of the honeys are classified as good category. Kars is an important province for beekeeping with floral variety. As a result of this study, the first step of the geographical marking studies of Kars' honey was completed.

Keywords: *Kars, Melissopalynology, Honey, TPN-10*

Kars Balının Coğrafi İşaretlemesi İçin Melissopalinolojik Analiz

Özet

Bu çalışmada, Türkiye'nin Doğu Anadolu Bölgesi'nde bulunan Kars İli'nde üretilen balların coğrafi işaretlenmesi için gerekli bir aşama olan melissopalinolojik analizleri yapılmıştır. Bu kapsamda sekiz ilçeden, örnekleme metoduna göre yapılan istatistiksel analizlerle tespit edilen 100 bal örneğinin mikroskobik analizleri gerçekleştirilerek Kars balına kaynaklık eden nektarlı bitkiler tespit edilmiştir. Bu amaçla melissopalinolojik analizleri yapılan 100 adet örnek balda; Apiaceae, Asteraceae, Berberidaceae, Betulaceae, Brassicaceae, Boraginaceae, Campanulaceae, Caryophyllaceae, Chenopodiaceae, Cistaceae, Cyperaceae, Dipsacaceae, Ericaceae, Fabaceae, Iridaceae, Lamiaceae, Liliaceae, Malvaceae, Onagraceae, Papaveraceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rhamnaceae, Rosaceae, Rubiaceae, Rutaceae, Salicaceae ve Scrophulariaceae familyalarına ait taksonların polenleri değişik oranlarda tespit edilmiştir. Fabaceae familyasından *Lotus corniculatus* (99 örnek), *Onobrychis radiata* (99 örnek), *Trifolium nigrescens* (88 örnek), Boraginaceae familyasından *Echium vulgaris* (81 örnek) ve *Myosotis lithoospermifolia* (15 örnek) taksonlarına ait polenlere hemen hemen tüm bal örneklerinde rastlanılmış (dominant, sekonder, minör, eser) olmakla birlikte bu türler içinde de en yoğun olarak *Onobrychis radiata* polenleri gözlenmiştir. Ayrıca, melissopalinolojik analizler sırasında, ballarda polen teşhisinin yanı sıra 10 gram baldaki toplam polen sayısı (TPS-10) değerleri de hesaplanmıştır. Hesaplamalar sonucunda minimum: 226, maximum: 481157 ve ortalama: 31678 TPS-10 değerleri elde edilerek balların polence zenginlikleri belirtilmiştir. Sonuç olarak, bu çalışma ile arıcılık için floral zenginliğiyle önemli bir il olan Kars'ın ballarına ait coğrafi işaret çalışmalarının ilk basamağı gerçekleştirilmiştir.

Anahtar sözcükler: *Kars, Melissopalinoloji, Bal, TPS-10*

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INTRODUCTION

Honey is a unique food product consisting of carbohydrates, amino acids, proteins, organic acids, vitamins, minerals and various phytochemicals. It is produced by bees from the nectar collected from a large variety of flowers, and its chemical composition, physical, sensory and biological properties depend on the nectar source [1]. Honey bees select their forage plants primarily on the basis of the sugar content of the plant nectar which is the raw material of honey [2].

Melissopalynology is of great importance for quality control of honey. Honey always includes numerous pollen grains and honeydew elements, so these contents provide a good fingerprint of the environment where honey comes from. Pollen analysis can therefore be useful to determine and control the geographical and botanical origin of honeys [3]. Multifloral honey can never be derived from a single botanical source. On the contrary, the term "unifloral" honey is used to describe honey produced mostly from one species. Generally, the pollen content for a honey to be called "unifloral," the percentage should be at least 45% of the total pollen count [4].

Due to the location of Turkey, different climatic conditions and plant cover can be observed in this country. Turkey includes three phyto-geographical and seven geographical regions. Turkey has a rich and interesting floristic structure. It has more than 10.000 plant species naturally and culturally grown and nearly 450 species are nectary plants which are known to be important in apiculture [5]. There are 9222 naturally grown species in Turkey and 3.000 of these are endemic [6]. Because of its climatic conditions and flora, Turkish honey is quite valuable.

Turkey has an important place among honey producing countries in the world. In Turkey, production of honey amounted to 105727 tons in 2016 (http://www.tuik.gov.tr). Kars is located in East Anatolia region of Turkey and also beekeeping in Kars is over average in Turkey's ratings of honey production per hive.

Pollen analysis of Turkish honey was firstly done by Sorkun and İnceoğlu [7]. Subsequently, more research about microscopic analysis of Turkish honey was carried out by other researches parallel to world literature [7-11]. By this study, we aimed to analyse honey samples produced in Kars to make geographical marking of Kars honey. These results will be a step towards further studies.

MATERIAL and METHODS

Collection Of Plant Materials for Reference Pollen Slides

In field study, 138 plants were collected from surrounding beehives that honey samples are collected from. After the identification of plants, pollen slides of these plants were prepared as reference slides.

Statistical Methods

Firstly, all the number of stable beehives in Kars were determined. It was observed that 399 beehives are stable in Kars region. Random sampling method were used to determine the number of beehives to collect honey samples instead of collecting from all 399 beehives. According to the statistical results analyzing 100 samples of were sufficient to form an opinion about Kars honey.

Collection of Honey Samples

Honey samples were collected from eight towns of Kars. The number of beehives for each town, that the samples were collected from, are determined according to the random sampling method-statistical analysis. The towns and the samples collected from them are given in *Fig. 1*.

Preparation of Pollen Slides for Botanical Origin

The floral sources of honey samples were determined by the mellisopalynological method. The materials were prepared for examination under the microscope according to the method of Louveaux et al.[12] and Sorkun [13]. Accordingly, 10 g of stock honey samples thoroughly mixed with a sterile glass rod were taken and transferred to the test tube and then 20 mL of distilled water was added. For dissolution of the honey sample in water, the tubes were placed in a water bath at about 45°C for 10-15 min and then each tube was shaken by a stirrer. The solution is then centrifuged at 3500 rpm for 45 min and the supernatant fraction is poured off. The precipitate remaining at the bottom of the tube was infused with a quantity of basic-fucose added glycerin-gelatin taken from the needle tip, and this material was then transferred onto the slide. The slide was heated at 30-40°C to allow the dissolution of basic fuchsine, and was added glycerin gelatin. Then, 18x18 lamella was covered on top of it. The preparation was left to stand for about 12 h upside down, and then it became available for examination under microscobe. In the diagnosis of pollen grains, the microphotographs of pollens in literature and reference preparations were used [13]. And then, observed pollen types were classified into four categories: dominant pollen (≥45%, D), secondary pollen (16-44%, S), important minor pollen (>3-15%, M) and rare polen (3%<). When one pollen type represented >45% of the total number of pollen grains, the sample was classified as a monofloral honey [14]. Besides the determination of botanical origin, the total pollen number (TPN-10) of all samples were calculated according to the Moar [15].

Preparation of Slides for Total Number of Pollens

In order to determine the Total Number of Pollen types (TNP in 10 g honey), pollen preparations were prepared according to the method that was described by Sorkun

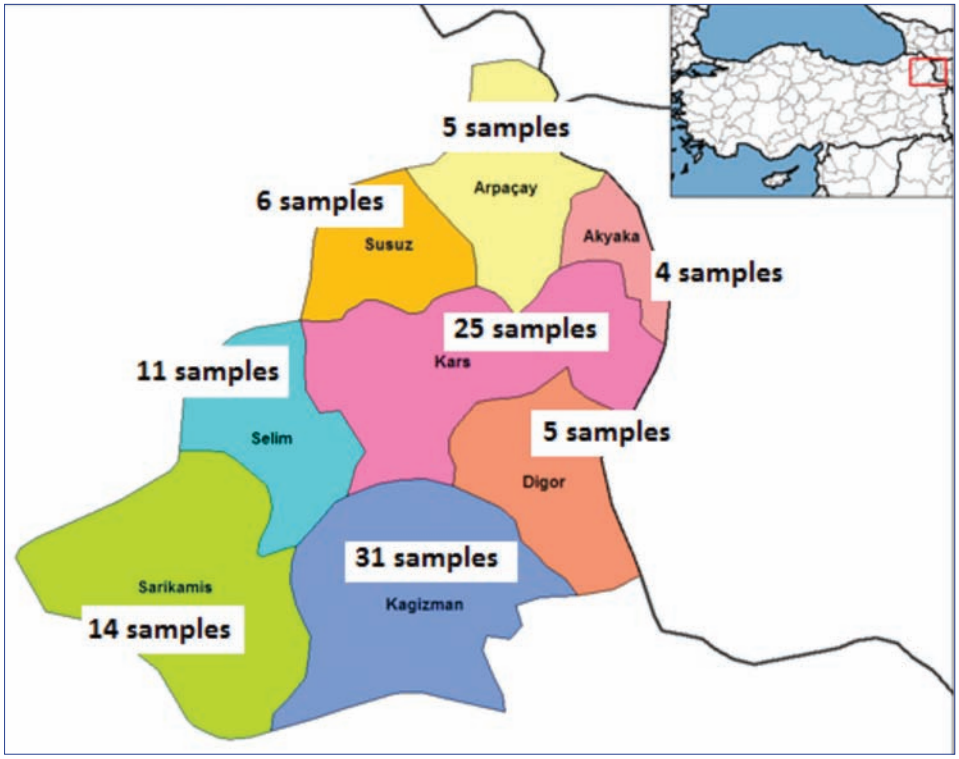

Fig 1. Map of Kars province (http://tr.wikipedia.org/wiki/Kars_(il) 2014)

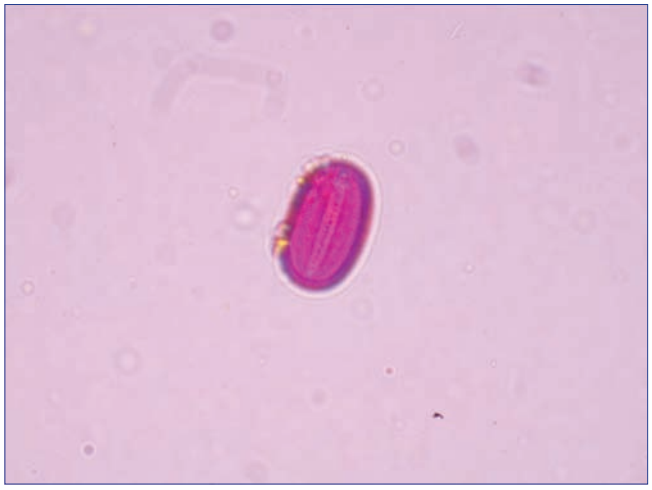

Fig 2. Microphotograph of *Onobrychis radiata* pollen

and Dogan [16]. According to this, 10 g from the stock honey was homogenized by mixing it thoroughly with a sterile glass rod. Then, 20 mL distilled water was added and a tablet containing 12542 *Lycopodium* spores was also put into the tube to control. After the tablet dissolved in the water, the tube was centrifuged at 3500-4000 rpm for 30 min. And then, the supernatant liquid was then poured off. To strain the water completely out of the tubes, the tubes were turned upside down onto a drying paper. Glycerine and precipitate were mixed homogeneously by adding 0.1 mL 50% of glycerine and a very little amount of bazic fuksin into the tube. 0.01 mL was taken from this mixture and put on a microscope slide, and the material was covered with 18x18 mm^2 of lamella. And then, the TNP-10 g preparations were examined under a light microscobe. At this stage, 10X objective was used for pollen counting. Finally, pollen classifications were made according to Moar et al.[15] and Maurizio and Hodges [17].

RESULTS

The pollens of the plants belonging to the family Apiaceae, Asteraceae, Berberidaceae, Betulaceae, Brassicaceae, Boraginaceae, Campanulaceae, Caryophyllaceae, Cheno podiaceae, Cistaceae, Cyperaceae, Dipsacaceae, Ericaceae, Fabaceae, Iridaceae, Lamiaceae, Liliaceae, Malvaceae, Onagraceae, Papaveraceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rhamnaceae, Rosaceae, Rubiaceae, Rutaceae, Salicaceae and Scrophulariaceae were found at different rates in the honey samples of the Kars region. Especially, pollens belonging to Fabaceae, Boraginaceae and Asteraceae families were frequently observed in honey samples. Pollens belonging to *Lotus corniculatus, Onobrychis radiate*, *Trifolium nigrescens* from Fabaceae family, *Echium vulgaris* and *Myosotis lithoospermifolia* from Boraginaceae family were observed frequently (dominant, secondary, minor, rare) nearly in all the investigated samples. The microphotograph of *Onobrychis radiata* pollen is shown in *Fig. 2*.

The pollen of the following taxa was found in the samples; *Carum* spp., *Eryngium billardieri*, *Malabaila dasyantha* from Apiaceae; *Achillea* spp., *Carduus nutans*, *Centaurea depressa*, *Centaurea triumfetti*, *Tussilago* spp., *Xanthium* spp., *Taraxacum* spp. from Asteraceae; *Sisymbrium elatum*, *Sinapis arvensis* from Brassicaceae; *Echium vulgaris*, *Cerinthe minör*,

Myosostis lithoospermifolia, *Rindera lanata*, *Silene vulgaris* from Boraginaceae; *Scabiosa columbaria* from Dipsacaceae; *Astragalus* spp., *Astragalus lagurus*, *Coronilla varia*, *Hedysarum* spp., *Lotus corniculatus*, *Medicago falcata*, *Trifolium repens*, *Trifolium nigrescens*, *Onobrychis radiata*, *Vicia sativa*, *Melilotus officinalis*, *Trifolium pratense*, *Trifolium ochrleucum*, *Onobrychis oxyodonta*, *Onobrychis tournefortii*, *Onobrychis* spp., *Lathyrus rotundifolius* from Fabaceae; *Iris* spp. from Iridaceae; *Salvia* spp., *Teucrium chamaedrys*, *Teucrium orientalis*, *Thymus longicaulis*, *Teucrium* spp., *Teucrium polium* from Lamiaceae; *Allium* spp. *Ornithagalum* spp. from Liliaceae; *Epilobium* spp. from Onagraceae; *Plantago lanceolata* from Plantaginaceae; *Rumex* spp. from Polygonaceae; *Nigella arvensis*, *Consolida orientalis* from Ranunculaceae; *Galium* spp. from Rubiaceae; *Salix* spp. from Salicaceae; *Linaria genistifolia* from Scrophulariaceae.

In short, the pollens identified by microscopic analysis of honey samples reflect the flora of Kars city. Plus, it is observed that the plants collected from the surroundings of the beehives show a resemblance with the melisso-playnological results.

TPN-10 values were calculated after mellisopalynological analysis and 226 was found as minimum, 481157 as maximum, 31 678 as mean value. The TPN-10 values and groups of honey samples are presented in *Table 1*. Classification of honey samples according to TPN-10 values was done according to Maurizio [18]. Accordingly, honey samples based on TPN-10 values were classified as group I (<20.000 pollen grains per 10 g honey), group II (20.000-100.000 pollen grains per 10 g honey), group III (100.000-500.000 grains per 10 g honey), group IV (500.000 -1.000.000 grains per 10 g honey), group V (>1.000.000 grains per 10 g honey). Also, honeys with very low pollen content, normal-pollen honeys and honeys with very rich pollen, were included in Group I, Group II and Group III, respectively [19].

DISCUSSION

As a result of the melissopalynologic analysis, it is possible to determine from which plants the honey is produced. In our study, as a result of the melissopalynologic analysis, 54 plant taxa belonging to 30 families were diagnosed in honey samples at different rates in the honey samples of the Kars region. Especially, pollens belonging to Fabaceae, Boraginaceae and Asteraceae families were frequently observed in honey samples. Consequently, important information on the nectar resources of the region has been obtained. These results indicate that honey samples from Kars are highly varied in terms of pollen content. It was an expected result that there was to be a lot of pollen diversity in honey samples from Kars province due to its climate, geographical position and rich plant cover of this region. Of the 100 samples analyzed, 21 were identified as unifloral and 79 as multiforal honey. Also, the pollens from

Table 1. *TPN-10 values of honey samples*

Town	Sample No	TPN-10	Groups
AKYAKA	28	25808	II
AKYAKA	53	9648	I
AKYAKA	54	4515	I
AKYAKA	60	15869	I
ARPAÇAY	9	481157	III
ARPAÇAY	27	26517	II
ARPAÇAY	39	24770	II
ARPAÇAY	64	25681	II
ARPAÇAY	65	55231	II
DİGOR	1	42303	II
DİGOR	10	5664	I
DİGOR	35	17311	I
DİGOR	46	22804	II
KAĞIZMAN	5	10034	I
KAĞIZMAN	19	6394	I
KAĞIZMAN	42	28920	II
KAĞIZMAN	43	10033	I
KAĞIZMAN	44	10750	I
KAĞIZMAN	45	36058	II
KAĞIZMAN	47	8026	I
KAĞIZMAN	48	29045	II
KAĞIZMAN	49	17366	I
KAĞIZMAN	50	16723	I
KAĞIZMAN	51	21038	II
KAĞIZMAN	52	17917	I
KAĞIZMAN	55	15305	I
KAĞIZMAN	56	7066	I
KAĞIZMAN	57	16461	I
KAĞIZMAN	58	58909	II
KAĞIZMAN	59	11208	I
KAĞIZMAN	87	23383	II
KAĞIZMAN	88	30691	II
KAĞIZMAN	89	17482	I
KAĞIZMAN	90	9345	I
KAĞIZMAN	91	16230	I
KAĞIZMAN	92	11328	I
KAĞIZMAN	93	6601	I
KAĞIZMAN	94	8466	I
KAĞIZMAN	95	16917	I
KAĞIZMAN	96	24883	II
KAĞIZMAN	97	20784	II
KAĞIZMAN	98	20381	II
KAĞIZMAN	99	6532	I
KAĞIZMAN	100	15241	I
MERKEZ	2	41807	II

Table 1. *TPN-10 values of honey samples (Continue)*

Town	Sample No	TPN-10	Groups
MERKEZ	3	2675	I
MERKEZ	4	46063	II
MERKEZ	11	226	I
MERKEZ	12	14165	I
MERKEZ	13	11825	I
MERKEZ	14	10091	I
MERKEZ	18	4561	I
MERKEZ	21	19462	I
MERKEZ	22	12425	I
MERKEZ	24	8710	I
MERKEZ	25	8361	I
MERKEZ	29	51804	II
MERKEZ	30	28832	II
MERKEZ	33	55635	II
MERKEZ	67	37009	II
MERKEZ	68	13159	I
MERKEZ	69	33369	II
MERKEZ	70	14856	I
MERKEZ	71	31260	II
MERKEZ	72	140440	III
MERKEZ	73	85442	II
MERKEZ	74	8640	I
MERKEZ	75	134516	III
MERKEZ	76	20839	II
SARIKAMIŞ	6	19020	I
SARIKAMIŞ	16	16278	I
SARIKAMIŞ	34	75252	II
SARIKAMIŞ	36	33905	II
SARIKAMIŞ	77	32426	II
SARIKAMIŞ	78	38364	II
SARIKAMIŞ	79	25762	II
SARIKAMIŞ	80	34620	II
SARIKAMIŞ	81	47158	II
SARIKAMIŞ	82	44266	II
SARIKAMIŞ	83	23154	II
SARIKAMIŞ	84	10083	I
SARIKAMIŞ	85	929	I
SARIKAMIŞ	86	38382	II
SELİM	7	6055	I
SELİM	15	15402	I
SELİM	20	143774	III
SELİM	23	6482	I
SELİM	26	21395	II
SELİM	32	55635	II
SELİM	37	7378	I

Table 1. *TPN-10 values of honey samples (Continue)*

Town	Sample No	TPN-10	Groups
SELİM	41	27519	II
SELİM	61	11901	I
SELİM	62	11288	I
SELİM	63	13259	I
SUSUZ	8	28035	II
SUSUZ	17	6689	I
SUSUZ	31	44655	II
SUSUZ	38	35776	II
SUSUZ	40	22234	II
SUSUZ	66	11208	I

Lotus corniculatus, Onobrychis radiata, Trifolium nigrescens taxa of family Fabaceae and *Echium vulgaris* taxa of family Boraginaceae were frequently found in almost all honey samples (as dominant, secondary, minor and trace) and among these taxa, *Onobrychis radiata* pollens were the most intense. It can be said that the taxa, which are determined to be predominant in honey samples, play a very important role in the composition of honey.

In our study, the pollen of Fabaceae was detected at different rates in all of the samples, dominant in 16 samples. On the other hand, the pollens of *Lotus corniculatus* (in 1 sample), *Trifolium nigrescens* (in 3 samples) and *Onobrychis radiata* (in 12 samples) were determined as dominant. The pollen of *Onobrychis radiata* from Fabaceae was detected in 99 of 100 samples as dominant (in 12 samples) and secondary (in 48 samples). These results suggest that *Onobrychis radiata* pollen could be a marker for Kars honey. Also, we have found pollen of *Lotus corniculatus* from Fabaceae in 99 samples as dominant (in 1 sample), secondary (in 38 samples), minor (in 50 samples) and rare (in 10 samples). *Trifolium nigrescens* pollen was detected in 80 samples as dominant (in 3 samples) and secondary (in 7 samples). In addition, pollens of *Astragalus* spp., *Astragalus lagurus, Coronilla varia, Hedysarum, Lathyrus rotundifolius, Medicago falcata, Medicago sativa, Melilotus officinalis, Trifolium ochrleucum, Onobrychis spp., Onobrychis tournefortti, Trifolium repens, Trifolium pratense, Onobrychis oxyodonta* and *Vicia sativa* taxa were found as secondary, minor and rare. Similary, Silici ve Gökçeoğlu [11] found that pollens of *Trifolium* spp. (in 3 samples) and *Astragalus* spp. (in 1 sample) were secondary in Antalya honeys. Plants such as *Trifolium, Lotus* (trefoil), and *Astragalus*, which have a long flowering period and are used as sources of pollen and nectar by bees, were also frequently observed.The results of our study indicate that these plants are also used as source of nectar in Kars region. On the other hand, in a different study it was reported that pollen of Fabacaea, *Castanea sativa* and Euphorbiaceae taxa were observed as secondary in honey samples from Kars region [19]. Contrary to these results, in our study, the pollen of *Castanea*

sativa and Euphorbiaceae taxa were not found in any sample. These results may be due to plant flora where the samples are collected from, which indicate that there is no distribution area of these plants in the regions where the samples examined in the scope of our study are collected.

We found pollens of Boraginace in all the samples. Pollens of *Echium vulgaris* (in 81 samples), *Cerinthe minör* (in 42 samples), *Myosotis lithoospermifolia* (in 15 samples), *Bunglossoides arvernsis* (in 8 samples), *Rindera lanata* (in 1 sample), *Anchusa* (in 1 sample) taxa from Boraginacaea found in honey samples. Among these, pollens of *Echium vulagris* (in samples 6, 37 and 72) and *Myosotis lithoospermifolia* (in samples 11 and 48) were detected as dominant.

From Asteraceae, pollen of *Achillea* spp. (in 24 samples), *Carduus nutans* (in 3 samples), *Centaurea depressa* (in 26 samples), *Centaurea triumfetti* (in 33 samples), *Tussilago* spp. (in 1 sample), *Xanthium* spp. (in 3 samples), *Taraxacum* spp. (in 8 samples) taxa were determined as minor and rare in honey samples. Similarly, many studies have been conducted to determine the origin of honey in samples collected from different origins [20, 21]. A group of researchers reported that they found pollens of plants belonging to the families Apiaceae, Asteraceae, Fabaceae and Rosaceae in honey samples as a result of their analysis of 25 honey samples [11]. In another study, it was reported that pollen of the *Hedera helix, Gossypium, Trifolium* (Kırıkkale), *Sophora, Rhododendron, Castanea sativa, Peganum harmala, Helianthus* taxa were identified as dominant in 13 floral honey collected from different regions of Turkey [22]. Similar to this study, we have detected pollen of *Trifolium nigrescens* was dominant in 3 honey samples. Temizer et al.[23] reported that Fabaceae taxa were common in honey samples collected from Giresun region. Similarly, Can et al.[24] reported that Fabaceae, *Trifolium* and *Rubus* taxa as predominant were found in honey samples collected from Kars province.

On the other hand, honey samples classified according to TPN-10 values. The TPN-10 value of 100 honey samples analyzed in the course of this study was determined between 226 and 481157. It was determined that 52 samples belong to group I (honey samples with low pollen content), 44 samples belong to group II (honey samples with pollen content at normal levels), and 4 samples belong to group III (honey samples with a very rich pollen content). It was detected that the honey sample with the least amount of pollen was sample 11 from Merkez (Group I) and the honey sample with the highest amount of pollen was sample 9 from Arpaçay (Group III). According to analysis carried out by Başoğlu et al.[25] on 25 honey samples collected from different regions of Turkey, it was detected that TPN-10 was between 400 and 12.400 in the 7 honey samples that were thought to be artificial, while it was between 14.800-37.800 in the 16 honey samples that were designated as pure honey. Moreover, it was found that TPN-10 was above the limit of 1.000.0000 in two honey samples, one of which was collected from Kars province. In another study, it was reported that the total number of pollen in two honey samples collected from Kars province was between 22713 and 6685 [19]. Similarly, in the study conducted by Sorkun and Doğan [16], it was reported that among 127 samples of natural flower honey samples collected from various regions of Turkey, TPN-10 was between 54383 and 38112 and TPN-10 in 42 artificial honey was between 954 and 4983. Researchers have reported that the value of TPN-10 in natural honey should be between 20.000 and 100.000 but this value can get below 20.000 in honey samples of Lamiaceae and Boraginacea families. Consistent with these results, we have determined that *Myosotis lithosppermifolia* (Boraginaceae) was the honey with the lowest TPN-10 value. Similar results with our study were also found in honey samples from diverse origins [23,26].

The honey samples were obtained from Kars province, located in Northeast Turkey and part of the Irano-Turanian phytogeographical region. The area is a pass between Caucasia and Anatolia. In addition, due to its geological, morphological and climatological differences, Kars region is also very rich in terms of plant diversity, which is the main source of beekeeping activities. For these reasons, it is not surprising that there is a rich content of honey samples produced in this region [27]. Sorkun and Yuluğ [9] also did melisopalynological investigations in this region with a narrower scope and found that *Onobrychis radiata* pollens are the most frequent plant. It is understood by this research that the 28 years of process between the two studies did not cause any serious change in the flora and vegetation.

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Conflict in Interest

The authors declare no competing financial interest.

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Research Article

Effect of Exogenous Fibroblast Growth Factor-21 on Inflammation in a High-fat Diet and Cholesterol Mice Model

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Abstract

Fibroblast Growth Factor 21 (FGF21) is a novel metabolic regulator involved in lipid utilization, and it can involve in the regulation of lipid metabolism. To determine the physiological function of FGF21 in relation to a high-fat diet (HFD) and high cholesterol, the effect of FGF21 on immune response indicators and hematological parameters was investigated. In the experiment, a total of 24 female mice were selected, 12 of which were fed with HFDs (group 1) and the other were fed with normal diet (group 2). After 30 days, the mice of two groups were respectively divided into two groups on average: 1. HFDs mice were used as model group; 2 normal diet mice were used as control group; 3. HFDs mice injection were used as model + FGF21 group; 4. normal diet injection were used as control + FGF21 group. Mice were sacrificed at 3-day intervals and the livers were isolated and analyzed. Blood was collected and analyzed for CD3 and CD19 by flow cytometry and IL-4, IL-6 and TNF-α by ELISA. Serum TG, TC, NEFA, LDL-c, and HDL-c levels were measured by automatic biochemical analyzer. Although CD3 and CD19 varied, the difference was not significant, and the levels of IL-4, IL-6, TNF-α, TC, TG LDL-c, and NEFA in the model exogenous injection FGF21 group were lower than in the model group ($P<0.05$). The present results indicate that exogenous FGF21 regulates IL-4, IL-6 and TNF-α and protects the liver from inflammation damage induced by high dietary fat and cholesterol.

Keywords: *Exogenous FGF21, High-fat diets, Cholesterol, Immunological factor*

Yüksek Yağlı Diyet ve Kolesterolle Beslenen Farelerde Eksojen Fibroblast Büyüme Faktörü-21'in Yangı Üzerine Etkisi

Özet

Fibroblast Büyüme Faktörü 21 (FGF21) yağların kullanımında görev alan metabolik bir düzenleyicidir ve yağ metabolizmasında rol oynar. Yüksek yağlı ve kolesterollü diyet ile FGF21'in fizyolojik fonksiyonu arasındaki ilişkiyi araştırmak amacıyla FGF21'in bağışıklık cevap indikatörleri ve kan parametreleri üzerine etkisi incelendi. Çalışmada toplam 24 dişi fare kullanıldı ve bunların 12'si yüksek yağlı diyet (Grup 1) diğer 12'si ise normal diyet (Grup 2) ile beslendi. Otuz gün sonunda iki gruptaki fareler tekrar ikişer gruba bölündü; 1: Yüksek yağlı diyet ile beslenen fareler "model grup" olarak kullanıldı, 2: Normal diyet fareler "kontrol grubu" olarak kullanıldı; 3: Yüksek yağlı diyet ile beslenen ve FGF21 enjekte edilen fareler "model + FGF21 grubu" olarak kullanıldı; 4: normal ile beslenen ve FGF21 enjekte edilen fareler "kontrol + FGF21 grubu" olarak kullanıldı. Fareler 3 gün aralıkla öldürüldü ve karaciğer dokuları incelendi. Kan toplanarak CD3 ve CD19 akışkan sitometresi ve IL-4, IL-6 ve TNF-α ise ELİSA ile analiz edildi. Serum TG, TC, NEFA, LDL-c ve HDL-c seviyeleri otomatik biyokimyasal analizleyici ile ölçüldü. CD3 ve CD19 değişmekle birlikte değişim istatistiki bakımdan anlamlı bulunmadı ve model eksojen enjeksiyon FGF21 grubunda IL-4, IL-6, TNF-α, TC, TG LDL-c ve NEFA seviyeleri model grubundan daha düşük olarak tespit edildi ($P<0.05$). Mevcut çalışmanın sonuçları eksojen FGF21'in IL-4, IL-6 ve TNF-α ekspresyonlarını düzenlediğini ve yüksek diyetsel yağ ve kolesterol ile oluşturulan karaciğer yangısal hasarına karşı organı koruduğunu göstermektedir.

Anahtar sözcükler: *Eksojen FGF21, Yüksek yağlı diyet, Kolesterol, İmmunolojik faktör*

INTRODUCTION

High-fat diets (HFDs) can increase the risk of many metabolic diseases, such as insulin resistance, hypertension, hyperlipemia, and liver disease [1]. HFDs is also closely related to metabolic inflammation, especially in the liver [2,3]. Liver

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is an important organ that regulates the energy homeostasis of the body [4]. Liver steatosis is not only confined to liver damage, but also closely associated with disorders of glucolipid metabolism [5].

A HFDs causes resistance of skeletal muscle glucose transport to insulin and contractions. HFDs-fed mice show glucose intolerance and decreased insulin sensitivity, accompanying by impaired insulin signaling. A HFDs can affect immunity functions and energy metabolism in the body. HFDs cause chronic inflammation, potentially damaging the liver's innate immune system. The abnormalities of the hepatic innate immune system (macrophages, neutrophils, and natural killer T cells) lead to the increased production of inflammatory cytokines, which contribute to the chronic inflammatory state of liver injury [6]. Inflammatory stress is closely related to metabolic disease and insulin resistance, implying that chronic inflammation accelerates the deterioration of B cell function. There are a number of ways to regulate the energy disorders caused by HFDs, such as reducing FFA production, inhibiting TNF-α production and its activity, inhibiting or inhibiting oxidative stress, triglyceride (TG) synthesis, and hepatic stellate cell activation. However, fibroblast growth factor (FGF21) have a variety of adverse effects or contraindications and there is still no consensus regarding the most effective drug therapy for liver steatosis. Therefore, new agents such as active endogenous molecules with high efficacy and minimal side effects are eagerly needed for the treatment of liver steatosis. FGF21 is a hepatic protein that plays a critical role in metabolism, stimulating fatty acid oxidation and liver uptake of fat [7]. Systemic metabolize of obese rodents adjusted by FGF21 leads to improved glucose homeostasis and weight loss. The concentration of immunity factors reduced and resulted in blood glucose and lipid levels decreased in the fatty rats by administrating FGF21, it proved that FGF21 can improve the metabolic disorders [8,9]. FGF21 changes the degree of inflammation of the liver caused by high fat, indirectly affecting the immune level changes. FGF21 plays an important role in anti-inflammatory responses induced by HFDs in mice. However, the underlying anti-inflammatory mechanism remains to be elucidated. In this study, we injected recombinant FGF21 into mice to evaluate the effect of exogenous FGF21 on the immunocytokines in mice fed a HFDs to determine the effect on inflammation.

MATERIAL and METHODS

Ethics

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC). All mice experimental procedures were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the school Council of Heilongjiang Bayi Agricultural University of China daqing.

Animals

Twenty-four female 4 weeks old KM mice (15-20 g) were purchased from Yongquan Animal Experiment Center, Heilongjiang, China. Twelve mice were fed a HFDs (feed formula: 10% load, 2% bilineurine, 5% albumen powder, 0.3% pig bile salt, and 82.7% basal feed), and the other twelve mice were fed a normal diet; mice were provided free access to the diet food and drinking water. After 30 days, the animals were divided into four groups: 1. HFDs mice below refer to 'model group' (n=6, intraperitoneal injection of 0.9% saline); 2. normal diet mice below refer to 'control group' (n=6, intraperitoneal injection of 0.9% saline); 3. HFDs mice exogenous injection FGF21 with 200 mg/kg/day FGF21 below refer to 'model + FGF21 group' (n=6, intraperitoneal injection); and 4. normal diet exogenous injection with 200 mg/kg/day FGF21 below refer to 'control + FGF21 group' (n=6, intraperitoneal injection). Mice were sacrificed at 3 and 6 days after injection. The experiment mice were lightly anesthetized and blood was collected from the retro-orbital plexus. The whole blood sample was then centrifuged at 2500 g for 10 min to yield the serum fraction and frozen at −20°C until it was needed for subsequent biochemical analyses. Blood was collected at 3 and 6 days, and after blood collection, the mice were sacrificed, and the liver was removed and fixed in buffered formalin for 24 h and embedded in paraffin wax for histological analyses.

Histological Analyses

The liver tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned (4 μm). The sections were stained with hematoxylin and eosin and observed under a microscope (Nikon, Tokyo, Japan).

Flow Cytometry

Whole blood was collected in anticoagulant tubes and the separation of lymphocytes from peripheral blood lymphocytes was performed (Solarbio, Beijing, China). Monoclonal antibodies against CD3 (SC-20047FITC, PC3/188A, Santa Cruz Biotechnology Inc) and CD19 (SC-8499PE, R20, Santa Cruz Biotechnology Inc) were used. The presence of immune T and B leukomonocytes in serum was analyzed by flow cytometry. For each analysis, 10.000 events were recorded [10].

ELISA Assay

Serum IL-4 and IL-6 levels were measured using a commercial ELISA kit (BOSTER, Wuhan, China) according to the manufacturer's guidelines. The optical density was determined at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The calibration curves

were constructed by plotting the net average absorbance of the standards on the Y-axis and the concentrations on the X-axis using the logit-log function to linearize and draw the best fitting curve.

Biochemical Index Examination

Serum total cholesterol TC, TG, and NEFA were measured by personnel at the University Hospital of Jilin according to routine clinical chemistry methods (Siemens, Erlangen, Germany).

Statistical Analysis

Data were pooled as mean values ± standard errors. The ANOVA corrected using the Bonferroni test was used to determine differences between groups comparing the internal variability of groups with the variability among all experimental groups. $P<0.05$ was considered statistically significant. Data were randomly collected and the analyses were performed using IBM SPSS Statistics 22.0 software (SPSS, Chicago, IL, USA).

RESULTS

To study the potential anti-obesity effect of exogenous recombinant FGF21, a HFDs model was successfully established. We first injected the FGF21 protein to HFDs mice after 30 days days via intraperitoneal injection. The daily doses were 200 mg/kg of FGF21.

Histological Analysis of Liver

Weight gain in HFDs feeding results from the accumulation of adipose tissue. Therefore we performed histological analyses of the liver. The livers of mice were collected after 6 days and analyzed. The number of vacuoles was lower in the model + FGF21 group than in the model group, and vacuole size decreased slightly *(Fig. 1c)*. The normal liver structure of the control group showed normal-sized liver cells, and the liver cord, nuclear circle, and cytoplasm were normal, neatly arranged and located in the central cells *(Fig. 1b)*. The degree of steatosis in the control + FGF21 group has no significant difference compared with the control group. These findings indicated that HFDs feeding resulted in significant hepatic steatosis and liver injury in mice. Based on the results of the NAFLD (nonalcoholic fatty liver disease) diagnostic gold standard, namely the histological analysis, these findings indicated that exogenous injection FGF21 had a significant hepatoprotective effect on HFDs-induced steatosis.

Changes of Cytokines and Biochemical Indices

Next, we investigated how FGF21 suppresses hepatic inflammation. FGF21 affected slightly increased cytokine-

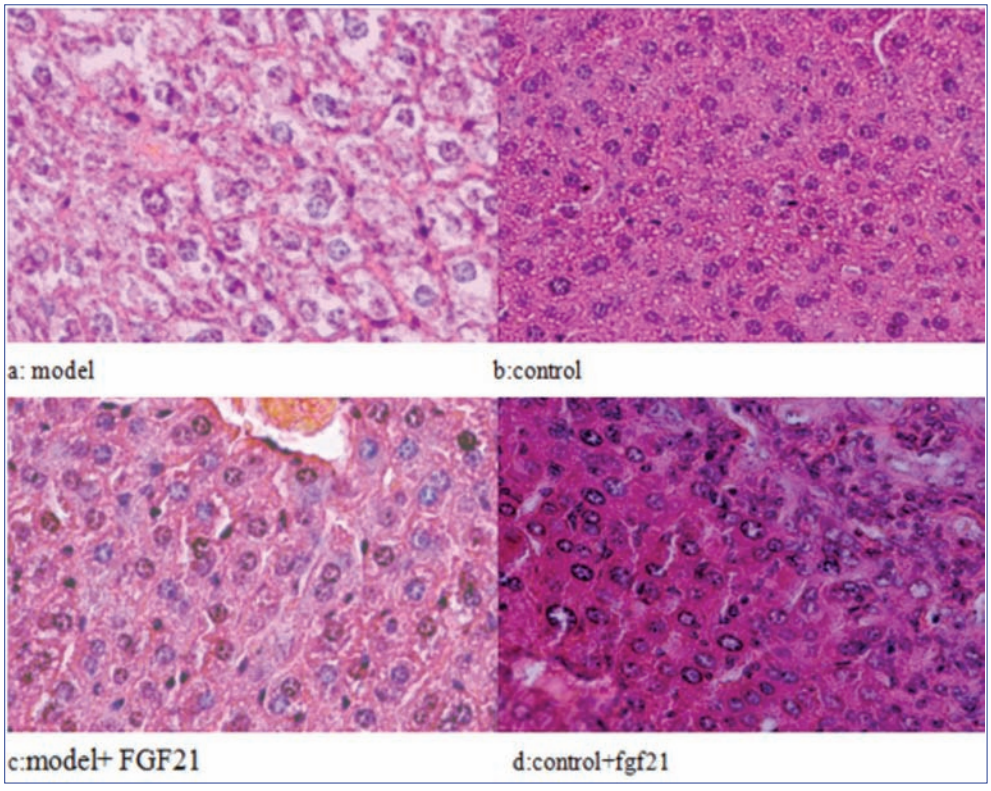

Fig 1. A HFDs caused liver pathological changes in exogenous injection FGF21 HDFs animals. Representative photomicrographs of liver sections stained with hematoxylin and eosin (200×). Notable differences in liver morphology are observed. After consuming a HDFs for 30 days, exogenous injection FGF21 HDFs mice (b) had reduce vacuolar changes compared with obese mice (a)

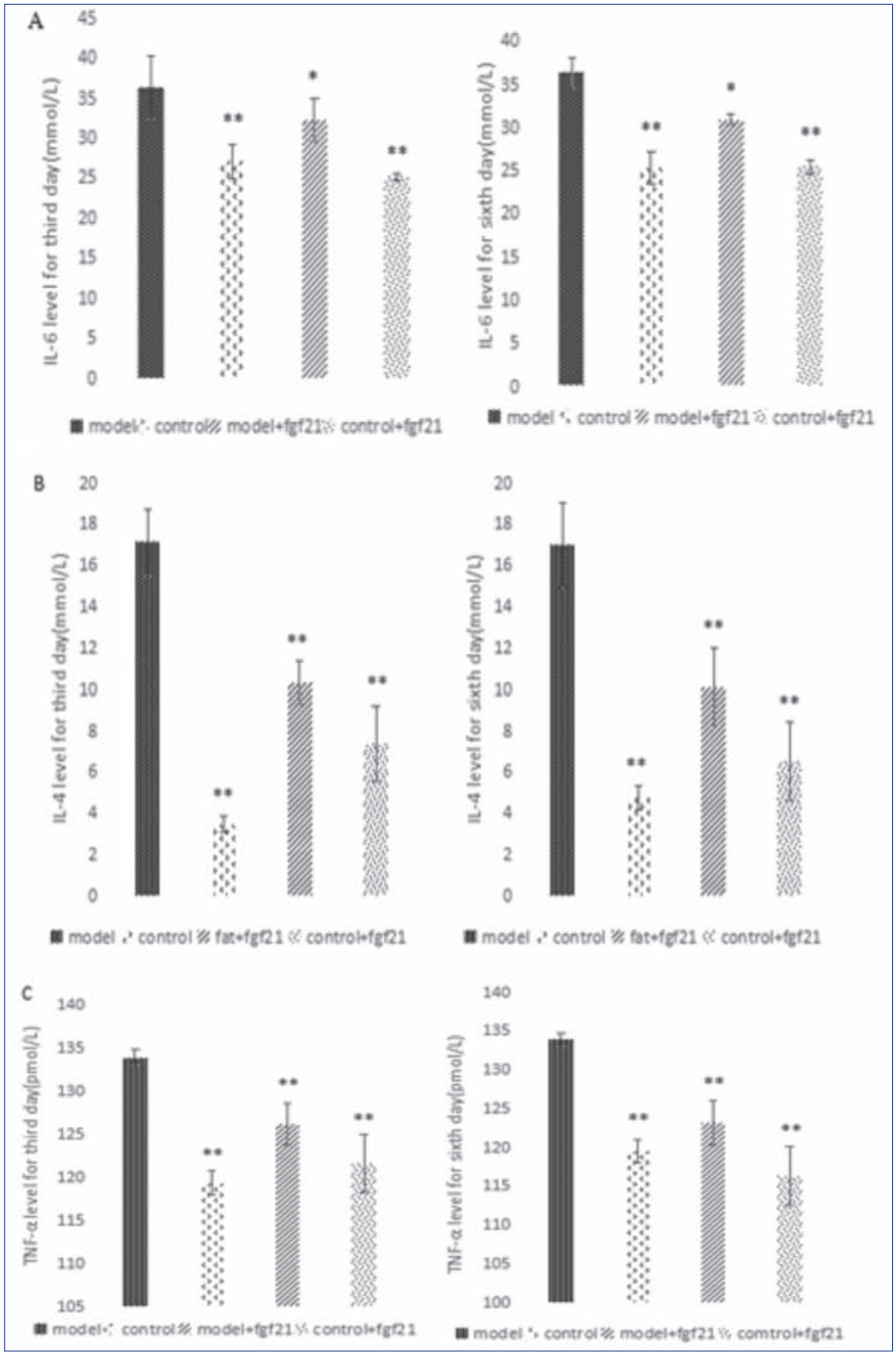

Fig 2. FGF21 reverses diet-induced obesity as indicated by immune factors and biochemical indicators. HDFs mice were subcutaneously injected with FGF21. The levels of IL-4 and IL-6 were measured at 3 and 6 days after exogenous injection FGF21. Data represent the mean ± SEM (three animals per group). All statistical comparisons were performed by one-way ANOVA followed by a multiple-comparison test. *P<0.05, **P<0.01 compared with the model group. A, The changes of serum IL-6 at 3 and 6 days. B, The changes of serum IL-4 at 3 and 6 days. C, The changes of serum TNF-α at 3 and 6 days

induced (i.e. TNF-α) inflammatory factor expression *(Fig. 2C)*. The changes of IL-4, IL-6, and TNF-α in each group are shown in *Fig. 2*. IL-6, IL-4, and TNF-α levels were significantly higher in the model group than in the model + FGF21 group at 3 and 6 days (P<0.01) *(Fig. 2A,B)*. There were no significant difference in IL-4, IL-6, TNF-α between the control group and the exogenous injection group.

TG levels were measured as the main NAFLD markers biochemical indicator. On days 3 and 6, TG was significantly lower in the model exogenous injection group, and on day 3, the model group and the control group were very significantly different (P<0.01). On days 3 and 6, LDL-c, TC, and NEFA had significantly different between model group and model exogenous injection FGF21 group. HDL-c was followed exogenous injection change. The above results indicate that FGF21 can significantly improve Lipid metabolism in KM HDFs mice.

After 3 days of exogenous injection, blood samples were collected for assessment of serum TC, TG, NEFA, LDL-C, and HDL-C levels using an automatic biochemical analyzer *(Table 1)*.

After 6 days of exogenous injection, blood samples were collected for assessment of serum TC, TG, NEFA, LDL-C and

***Table1**. Effect of FGF21 on the changes in serum lipid parameters at the third day*

Group	TG/mmol·L^{-1} (mean±SEM)	TC/mmol·L^{-1} (mean±SEM)	NEFA/mmol·L^{-1} (mean±SEM)	LDL-c/mmol·L^{-1} (mean±SEM)	HDL-c/mmol·L^{-1} (mean±SEM)
Model (n=3)	0.720±0.072	2.00±0.057	0.440±0.023	0.407±0.012	0.593±0.024
Control (n=3)	0.413±0.073**	1.20±0.050**	0.260±0.031**	0.237±0.027**	0.447±0.053
Model + FGF21 group (n=3)	0.567±0.024	1.67±0.037*	0.340±0.012*	0.297±0.019*	0.807±0.066*
Control + FGF21 group (n=3)	0.537±0.038*	1.23±0.043**	0.233±0.037**	0.287±0.024*	0.693±0.103

** P<0.05, ** P<0.01 vs. the model group*

***Table 2.** Effect of FGF21 on the changes in serum lipid parameters at the sixth day*

Group	TG/mmol·L^{-1} (mean±SEM)	TC/mmol·L^{-1} (mean±SEM)	NEFA/mmol·L^{-1} (mean±SEM)	LDL-c/mmol·L^{-1} (mean±SEM)	HDL-c/mmol·L^{-1} (mean±SEM)
Model (n=3)	0.673±0.077	1.99±0.068	0.426±0.017	0.400±0.023	0.587±0.035
Control (n=3)	0.521±0.029**	1.06±0.094**	0.220±0.020**	0.247±0.029**	0.467±0.038
Model + FGF21 group (n=3)	0.454±0.024*	1.57±0.037**	0.353±0.017*	0.300±0.002**	0.793±0.096*
Control + FGF21 group (n=3)	0.423±0.052**	1.26±0.219**	0.243±0.015**	0.220±0.058**	0.774±0.070*

** P<0.05, ** P<0.01 vs. the model group*

HDL-C levels using an automatic biochemical analyzer *(Table 2)*.

Different Exogenous Injection FGF21 Days Contast

Compared with the index of model + FGF21 and control + FGF21 on the third day, the index on the sixth day has no significant differences in IL-4, IL-6, TNF-α, TC, NEFA, LDL-c and HDL-c *(Fig. 3)*. But the trend of model exogenous injection group to the normal development in IL-4, IL-6, TNF-α, TC, NEFA, LDL-c and HDL-c.

In the model exogenous injection groups, the third day was compared TG, TC, NEFA, LDL-c and HDL-c with the sixth day *(Table 3)*.

In the control exogenous injection groups, the third day was compared was compared TG, TC, NEFA, LDL-c and HDL-c with the sixth day *(Table 4)*.

Immune Cells

Immune cells from blood of 30-days HDFs mice for 6 days were analyzed by flow cytometry. T lymphocytes (CD3 cells) and B lymphocytes (CD19 cells) showed no marked differences between fat exogenous injection and HDFs mice, and no differences were observed between the control and exogenous injection groups *(Fig. 4)*. In the HDFs mice, T (CD3) and B (CD19) lymphocyte numbers showed a similar profile in response to the nutritional challenge, i.e. HDFs does not cause a strong inflammatory response. However, CD3/CD19 values decreased significantly.

DISCUSSION

HFDs can cause chronic inflammation and potentially damage the liver's innate immune system in long - term edible. The liver is an important organ that plays a critical role in the innate immune system [11,12]. The inflammatory response is a complex reaction of the immune system. Activation of lymphocytes plays a pivotal role in the development and progression of inflammation. T and B lymphocytes play a major role in cellular and humoral immunity, in which CD3 cells represent the total number of T lymphocytes and CD19 is an important marker of B lymphocytes.

In our study, we found that FGF21 could improve lymphocytes to reduce inflammation. we investigated the effect of FGF21 on HFDs mice CD3 and CD19. The results showed that blood CD19 was increased in mice fed a HFDs. As the severity of NAFLD increased, CD19 tended to increase, indicating that humoral immune enhancement, which may be involved in the pathogenesis of NAFLD, may be caused by the production of excessive immunoglobulin. The increased percentage of CD3/CD19 cells may be the result of a decrease in the absolute number of other lymphocyte subsets.

In this study, we investigated the mechanism underlying the effect of FGF21 on inflammatory factors in mice fed a HFDs. The results showed that FGF21 effectively reduced TC and NEFA levels and alleviated hepatic damage. The lack of IL-6 expression was associated with exacerbated steatosis in HFD conditions. In our study,The level of serum IL-6 was higher in the model group than that in the control group. An important source of IL-6 in obesity is the expanding visceral adipose tissue mass. Regarding the hepatic lipid metabolism, there is evidence that IL-6 affects the opposing fatty acid pathways: degradation and Synthesis. The HFD-derived effects were aggravated in IL-6-deficient mice: higher cholesterol levels and decreased TG levels in serum. In the context of HFD-induced obesity, the

Table 3. *Exogenous FGF21 between model groups in serum lipid parameters at the third day and sixth day*

Group	TG/mmol·L^{-1} (mean±SEM)	TC/mmol·L^{-1} (mean±SEM)	NEFA/mmol·L^{-1} (mean±SEM)	LDL-c/mmol·L^{-1} (mean±SEM)	HDL-c/mmol·L^{-1} (mean±SEM)
Model + FGF21 group at third day (n=3)	0.567±0.024	1.67±0.037	0.340±0.012	0.297±0.019	0.807±0.066
Model + FGF21 group at sixth day (n=3)	0.454±0.024	1.57±0.037	0.353±0.017	0.300±0.002	0.793±0.096

** P<0.05, ** P<0.01 vs. the model+FGF21 group*

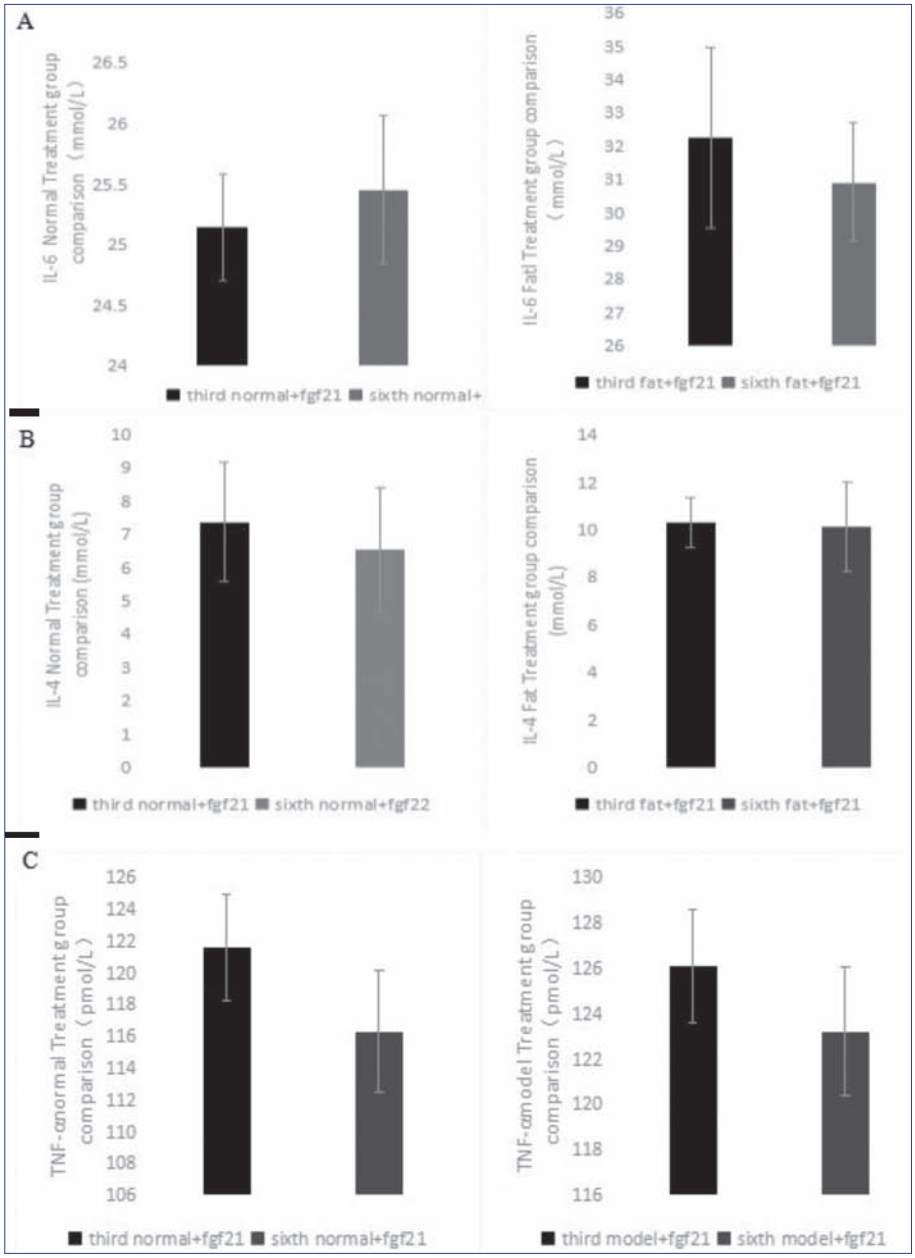

Fig 3. In the control exogenous injection and model exogenous injection groups, the third day was compared IL-4, IL-6 and TNF-α with the sixth day. Data represent means±SEM (three animals per group). All statistical comparisons were performed by one-way ANOVA followed by a multiple-comparison test. A, Comparison of the effects of exogenous injection FGF21 on IL-6 at 3 and 6 days. B, Comparison of the effects of exogenous injection on IL-4 at 3 and 6 days. C, Comparison of the effects of exogenous injection FGF21 on TNF-α at 3 and 6 days

administration of rIL-6 might contribute to the aggravation of fatty liver disease through increasing lipogenesis [13]. IL-6 is considered as an anti-inflammatory cytokine through its inhibitory effects on TNF-α [14,15]. FGF21 can effectively change the production of IL-6, thereby reducing the production of TNF-α. In previous studies, the functions of IL-4 were primarily investigated in the immune system, where it exerts pleiotropic effects ranging from Th2 differentiation of helper T cells, activation of B cells to release IgE, and stimulation of alternative macrophage activation [16,17]. IL-4 is usually higher in the early stages of inflammation. Because we established a HDFs model, IL-4 values may be high in the model group, however, the exogenous injection in the same period and the relative reduction in IL-4 may indicate a therapeutic effect. Elevated IL-4 levels in

Table 4. *Exogenous FGF21 between comtrol groups in serum lipid parameters at the third day and sixth day*

Group	TG/mmol·L^{-1} (mean±SEM)	TC/mmol·L^{-1} (mean±SEM)	NEFA/mmol·L^{-1} (mean±SEM)	LDL-c/mmol·L^{-1} (mean±SEM)	HDL-c/mmol·L^{-1} (mean±SEM)
Control + FGF21 group at third day (n=3)	0.537±0.038	1.23±0.043	0.233±0.037	0.287±0.024	0.693±0.103
Control + FGF21 group at sixth day (n=3)	0.423±0.052	1.26±0.219	0.243±0.015	0.220±0.058	0.774±0.070

** P<0.05, ** P<0.01 vs. the model + FGF21 group*

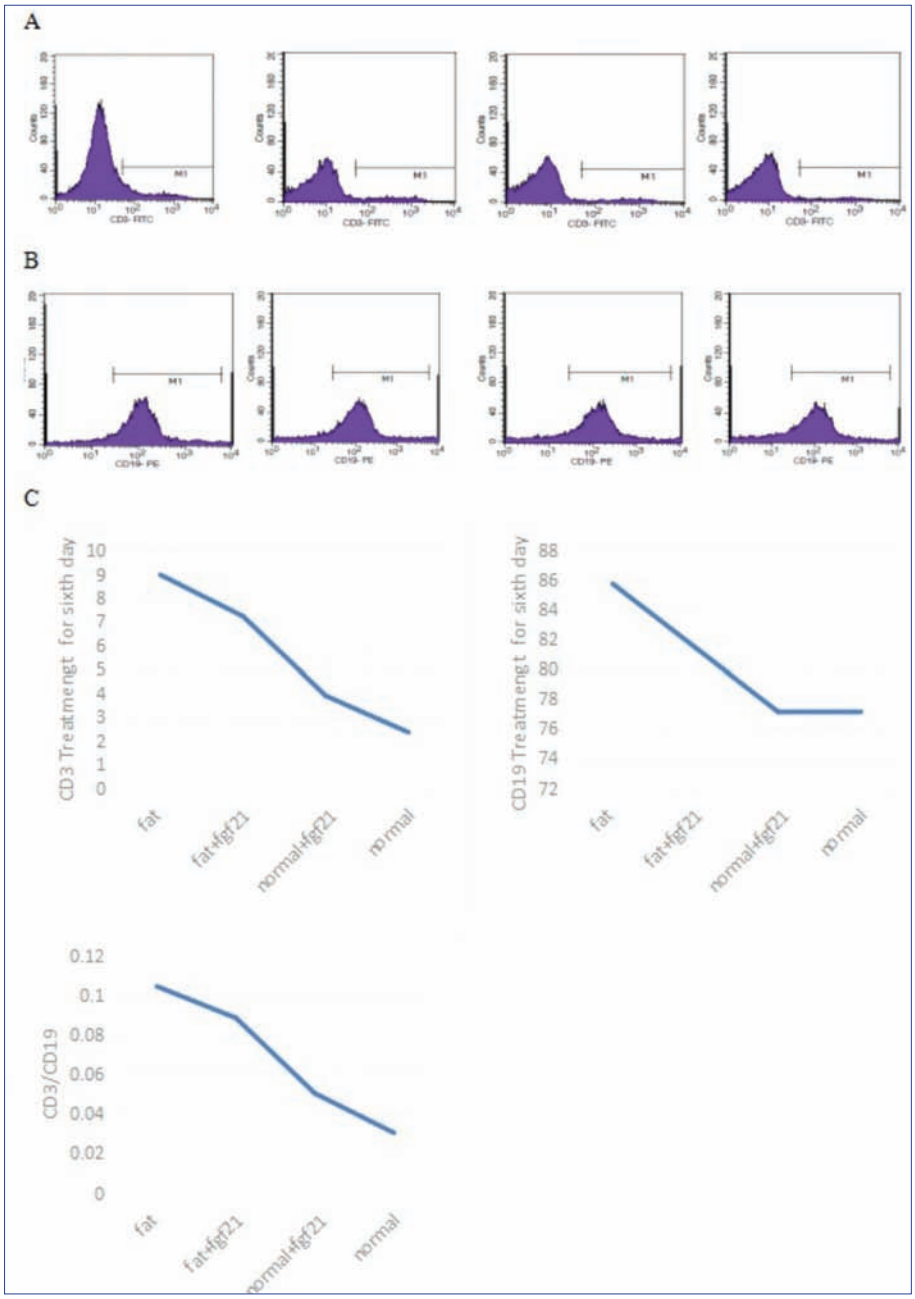

Fig 4. CD3 and CD19 were detected by flow cytometry. A, CD3 cell numbers by flow cytometry. B, CD19 cell numbers by flow cytometry. C, CD3, CD19 and CD3/CD19 linear analysis

the control + FGF21 group compared with that in the control group may be due to exogenous injection.

Our result indicated that the model + FGF21 group improved after exogenous injection on days 3 and 6. However, there were no significant differences between days 3 and 6 in the exogenous injection group, which may be due to the fact that the injection cycle was too short. The effects of FGF21 on decreasing body fat in model mice may have been mediated by FGF21-induced suppression of hepatic carbohydrate oxidation may occur via PDK4-mediated suppression of PDC activity [18]. A HFDs induced hepatic steatosis in mice, leading to increased TG and NEFA. This resulted in NAFLD leading to inflammatory

changes in the liver. IL-4, IL-6, and TNF-α were increased by different degrees. FGF21 effectively relieved hepatic fat deformation, inhibited the synthesis of NEFA and TG and reduced the inflammation caused by changes in the inflammatory factors IL-4, IL-6, and TNF-α, and thus alleviated inflammation. Induced by HFDs.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

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Research Article

The Anatomical and Histological Structures of Buzzard's *(Buteo buteo)* Small Intestine and Liver, and Immunohistochemical Localization of Catalase

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Abstract

This study aimed to examine the structure of the buzzard's *Buteo buteo)* small intestine and liver, and the localization of catalase in those organs. Crossman's modified triple stain and Periodic Acid Schiff (PAS) stain were applied to the samples for histological examination. To detect catalase localization in the tissues, the avidin-biotin-peroxidase complex (ABC) technique was used. It was observed that the general histological structure of the buzzard's small intestine and liver was similar to that of other avian species. Catalase immunoreactivity in the lamina propria and submucosa in the small intestine were also observed, as well as catalase immunoreactivity in the hepatocyte cytoplasm in the liver. Some areas in the small intestine and liver had catalase immunoreactivity, which means that an antioxidant defence occurs in those areas.

Keywords: *Antioxidant Enzyme, Buzzard, Catalase, Immunohistochemistry, Liver, Small intestine*

Şahin *(Buteo buteo)* İnce Bağırsak ve Karaciğerinin Anatomik ve Histolojik Yapısı İle Katalazın İmmunohistokimyasal Lokalizasyonu

Özet

Bu çalışmada şahinlerin *(Buteo buteo)* ince bağırsakları ile karaciğerlerinin genel yapısı ve bu dokularda katalazın immunohistokimyasal lokalizasyonunun incelenmesi amaçlandı. Histolojik incelemeler için dokulara üçlü boyama ve periyodik asit Shiff (PAS) boyaması yapıldı. Dokulardaki katalaz enziminin immunohistokimyasal lokalizasyonunun belirlenmesi için Avidin-Biotin-Peroksidaz Kompleks (ABC) tekniği uygulandı. Şahin ince bağırsağı ve karaciğerinin genel histolojik yapısı incelendiğinde diğer kanatlı türlerine benzer olduğu görüldü. Katalaz immunoreaktivitesinin ince barsaklarda lamina propria ve submukoza katmanlarında, karaciğerde ise hepatositlerin sitoplazmalarında olduğu görüldü. Antioksidan bir enzim olan katalazın ince bağırsak ve karaciğerde belirtilen yerlerde olması bu bölgelerin antioksidan savunmanın gerçekleştiği yer olduğunu düşündürmektedir.

Anahtar sözcükler: *Antioksidan Enzim, İmmunohistokimya, İnce bağırsak, Karaciğer, Katalaz, Şahin*

INTRODUCTION

The digestive system has many functions, such as digestion and absorption of nutrients, which are necessary for organisms [1]. The avian small intestine has three parts: the duodenum, jejunum and ileum, which makes it similar to that of mammalians [2]. The duodenum arises from the upper right side of the gizzard and forms a u-shaped fold in all avian species. The jejunum, which is the longest part of the small intestine, is situated in the lower right quadrant of the abdomen. The ileum, which is the shortest and thinnest part of the intestine, is located above the duodenum and below the rectum [3,4]. The structure of the avian intestine varies from one species to another. It is thought that those differences are based on varying diets in different species [5]. In carnivorous birds like the buzzard, the small intestine develops fully, but the cecum does not develop as well as the small intestine [6]. The wall structure of the small intestine in avians comprises tunica mucosa, submucosa, tunica muscularis and tunica serosa like that

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of mammalians [5]. Tunica mucosa is formed by leaf-shaped intestinal villi and the crypts of Lieberkuhn, which is short, simple and open between the villi and has tubular ducts. The epithelium consists of a single layer which includes three types columnar cells: main epithelial cells, goblet cells and enterochromaffin cells. Lamina propria consists of loose connective tissue which contains blood and lymph vessels, nerves and lymphoid cells. The lamina muscularis is not well-developed, and consists of longitudinal muscle fibres. The submucosa consists of connective and elastic fibres. The small intestinal submucosa of avians is thin, and includes many diffuse and nodular lymphocytes. The tunica muscularis consists of an inner circular and an outer longitudinal smooth muscle. Tunica serosa is a thin layer of loose connective tissue [2,5,7]. The avian duodenum does not contain Brunner glands, unlike the mammalian duodenum, which does contain them [2,8].

The liver is situated in the upper right quadrant of the abdominal cavity as two lobes; these are the left and right lobes, and the right lobe is larger than the left. There is a parenchyma bridge between the two lobes, which are separated from each other by the cranial interlobar fissure at the front and the caudal interlobar fissure at the back [2,9]. The avian liver is similar to that of mammalians in terms of histological features and functions. The structure of the interlobular septum and lobules are not seen in avian livers, but they are noticeable in mammalian livers [7,10,11]. However, each unit, which has central vein, is accepted as a lobule in avian livers [2]. In addition, there are no lymph nodes in avians (apart from some species), so there are lymph follicles and diffuse lymphoid tissue areas (lymphoid infiltration or ectopic lymphoid areas) in the liver, as in other organs (such as kidney, pancreas, gonads, thyroid and brain) [10,11]. Therefore it should not be taught those areas in avian species are caused only because of pathological reasons [7].

The digestive tract is always at risk of encountering pathogens, which cause various diseases, because it is directly open to the external environment. The effects of pathogens in the digestion system most probably affect other systems in an organism [12]. Reactive oxygen species, which are quite harmful for organisms, occur during normal functioning in the cell, or during inflammation caused by external pathogens in the digestive system. These harmful radicals cause various diseases. The organism protects itself against the harmful effects of reactive oxygen species with an antioxidant system, which includes antioxidant enzymes, such as catalase [13-15]. The amount of catalase in tissue, which protects the organism from harmful reactive oxygen species, is changeable [16].

This study aimed to investigate the anatomical and histological structure of the buzzard's liver and small intestine, as well as the immunohistochemical localization of catalase.

MATERIAL and METHODS

This study was approved by the Local Ethics Commission of Experimental Animals of Kafkas University (KAÜ-HADYEK/2014-36) and the Turkish Republic Ministry of Forest and Water Management (General Directorate of Nature Conservation and National Parks [Approval no: 02.06.2015/119062]).

In this study, the small intestines and livers of four adult buzzards (*Buteo buteo*) were used. The livers were weighed with an electronic scale (0.01 g, SF-550), and the diameter and length of the small intestines were measured with an electronic caliper (0.01 mm, BTS). The nomenclature used in this study was consistent with the Nomina Anatomica Avium [17].

The samples for histological examination were fixed in a 10% formalin solution, following a routine histological process and then embedded in paraffin. Sections of 5 µm thickness were taken from paraffin blocks. Crossman's modified triple stain was applied to the sections to examine the histological structure of tissues [18], and Periodic Acid Shiff (PAS) stain was applied to detect glycogen-rich areas [19].

The avidin-biotin-peroxidase complex (ABC) technique was applied to determine immunohistochemical localization of catalase enzyme in tissues [20]. Sections taken from paraffin blocks were deparaffinized and rehydrated. Subsequently, 3% H_2O_2 was applied to the sections to block endogenous peroxidase activity. The sections were incubated in citrate buffer (0.1 M, pH: 6.0) in the microwave (800 watt, 10 min) for antigen retrieval, and the samples were washed with phosphate buffer solution (PBS, 0.1 M, pH 7.2). After the samples had been incubated in the blocking buffer for 10 min, they were washed with PBS. Next, slides were incubated with anti-CAT (catalase) antibody (abcam, [ab1877], Cambridge MA, USA), which was diluted at 1:1000 for the liver and at 1:1500 for the small intestine, for an hour at room temperature, and they were then washed with PBS. Afterwards, biotinylated secondary antibody was applied to the samples for 30 min (Ultravision Detection System, Thermo Scientific, Fremont, USA). Then the samples were washed with PBS again and incubated with streptavidin horseradish peroksidaz (Ultravision Detection System, Thermo Scientific, Fremont, USA) for 30 min. After washing the samples, DAB (Thermo Scientific, Fremont, USA) was applied to them. Negative control was used to determine specific catalase immunoreactivity, and hematoxylin stain was used as a nuclear counter stain.

RESULTS

Anatomical Results

During examination of the small intestines, it was observed that the duodenum arises from the upper right side of the

gizzard and forms a U-shaped fold. It was also observed that the jejunum was in the right side of the abdomen and that the ileum was above the upper side of the duodenum *(Fig. 1)*. Measurements showed that the average length of the duodenum, jejunum and ileum were 127.67±1.71 mm, 651.11±13.59 mm and 118.70±3.10 mm, respectively, and their average diameters were 4.79±0.39 mm, 5.08±0.62 mm and 3.93±0.45 mm, respectively.

The anatomical examination also revealed that the liver comprised two lobes, which were the right and left lobes *(Fig. 1)*. The average weight of the right lobe was 8.11±0.29 g after removal of the vesica fellea, and that of the left lobe was 9.41±0.82 g. The right and left lobes of the liver were separated from each other by two fissures, one of which was a deep cranial interlobar fissure at the front, while the other was a caudal interlobar fissure at the back. Inspection revealed that the diaphragmatic surface (parietal surface) was convex and the visceral surface was concave. A ventricular impression and cardiac impression were noticeable on both lobes of the liver.

Histological Results

It was observed that the wall of the small intestine consisted of tunica mucosa, submucosa, tunica muscularis and tunica serosa layers *(Fig. 2a)*. The epithelial laminae of the tunica mucosa were formed with single-layered of columnar cells and goblet cells were found between them. The lamina propria included diffuse lymphoid tissue. It was observed that the lamina muscularis extended into the intestinal villi as a smooth, thin muscular layer. The submucosa was seen to be loose connective tissue, which included lymphoid tissue. The tunica muscularis had inner circular and outer longitudinal smooth muscle layers, and the tunica serosa was composed of loose connective tissue.

The lobules in the buzzard's liver were not noticeable when they were examined histologically because it was seen that there were no interlobular septum *(Fig. 3a)*. Examination showed that in the case of hepatocytes, their edge was evident, surrounding the central vein, and there were also sinusoids between hepatocytes. The hepatic artery, interlobular vein and a bile duct were observed in the Kiernan space (portal field) in the liver. In addition, lymphoid areas were found in the liver *(Fig. 3a)*.

A PAS positive reaction was detected in the goblet cells and in the submucosa of the small intestine *(Fig. 2b)*. It was also observed in some hepatocyte cytoplasm and in some areas of connective tissue in the liver *(Fig. 3b)*.

Immunohistochemical Results

It was identified that there was cytoplasmic catalase immunoreactivity in the lamina propria and submucosa layers of the duodenum, jejunum and ileum during examination of the small intestine *(Fig. 4a)*. However, this reaction was not found in the epithelial lamina and lamina muscularis layers in those parts of the small intestine *(Fig. 4a,b)*. Some crypt epithelial cells had a very weak reaction, but no reaction in other parts of the crypts.

The catalase immunoreactivity in the liver was diffuse and granular in the hepatocyte cytoplasm *(Fig. 5a,b)*. The reaction was generally in the cytoplasm, but in some hepatocytes, the reaction was seen in both the cytoplasm and nuclei. It was observed that the hepatocytes had varying degrees of immunoreactivity *(Fig. 5b)*. There was no catalase immunoreactivity in the areas of connective tissue, endothelial cells, Kupffer cells and bile duct of the liver.

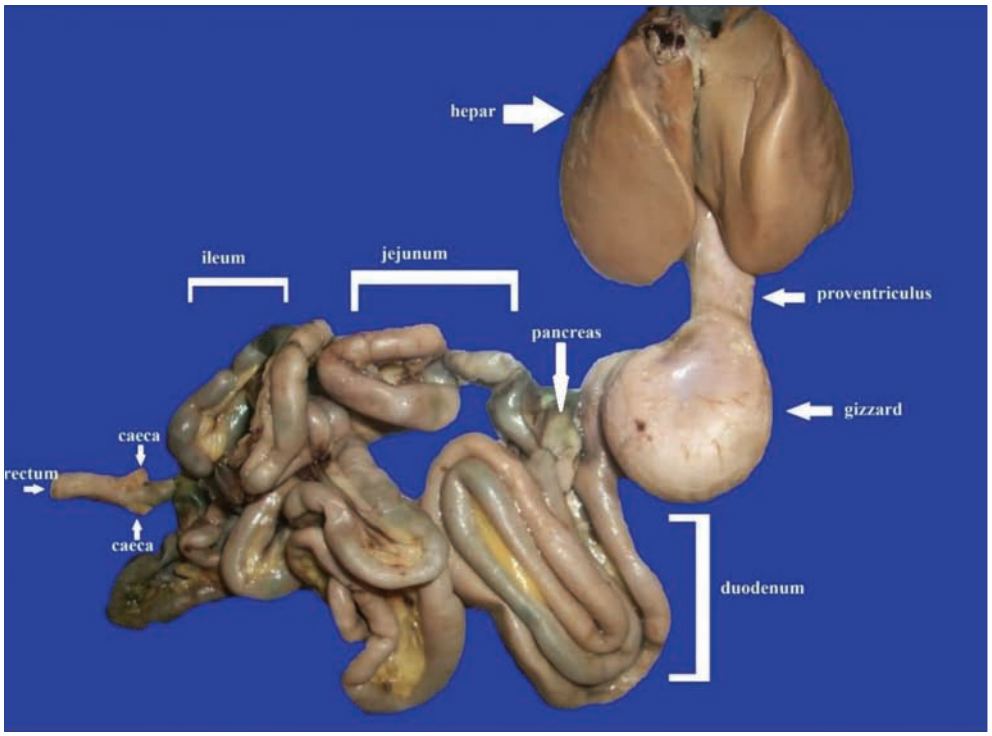

Fig 1. General view of Buzzard's digestive system

DISCUSSION

The purpose of this study was to examine the anatomical and histological structures of the buzzard's small intestine and liver, and also the localization of an antioxidant enzyme catalase in these organs.

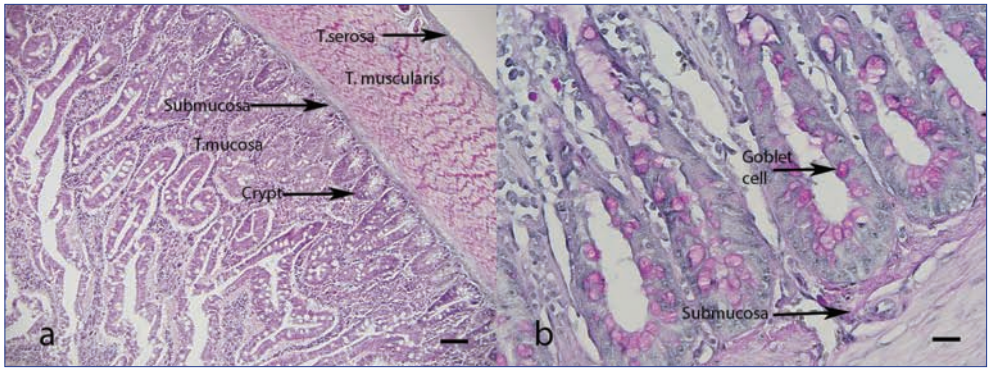

Fig 2. Small intestine. **a-** General view of small intestine, Crossman's modified triple staining, Bar: 200 µm; **b-** PAS staining in small intestine, Bar: 50 µm

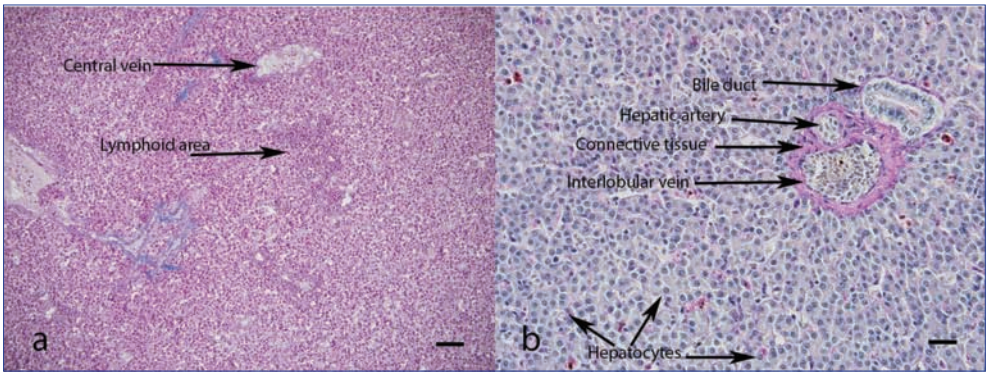

Fig 3. Liver. **a-** General view of liver. Crossman's modified triple staining, Bar: 200 µm; **b-** PAS staining in liver, Bar: 50 µm

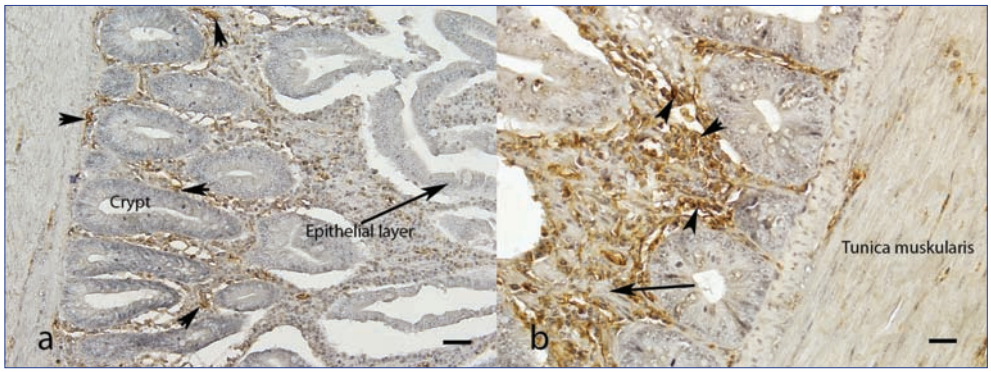

Fig 4. Catalase immunoreactivity in small intestine. **a-** *Arrow heads:* catalase immunoreactivity, Bar: 100 µm; **b-** *Arrow:* lamina muscularis, *Arrow heads: c*atalase immunoreactivity, Bar: 50 µm

The length and diameter of the small intestine vary from one species to another [2,21]. Anatomical examination of a buzzard's small intestine showed that the length and diameter of the duodenum, jejunum and ileum are similar to those of a pigeon [2,4]. The weight of the avian liver differs according to species, weight, race and diet [3,4]. It has been reported that the left lobe is larger than the right in chicken livers, but both are equal in turkey livers. Conversely, it has been noted that the right lobe is larger than the left in many avian species [3,4]. In this study, it was found that the left lobe was heavier than the right in the buzzard's liver.

During the histological examination, the wall of the small intestine comprised mucosa, submucosa, muscularis and serosa layers, and the structure of the epithelial cells, intestinal crypts and intestinal villi were similar to the data in the literature in terms of histological features that were consistent with other animals [2,7,22]. The absence of lobules in the liver, the hepatocytes surrounding the central vein, the structure of the portal triad in Kiernan's space and lymphatic tissue were in accordance with the existing literature [2,7,8,11]. In the current study, it was seen that there were lymphoid areas as infiltration in the

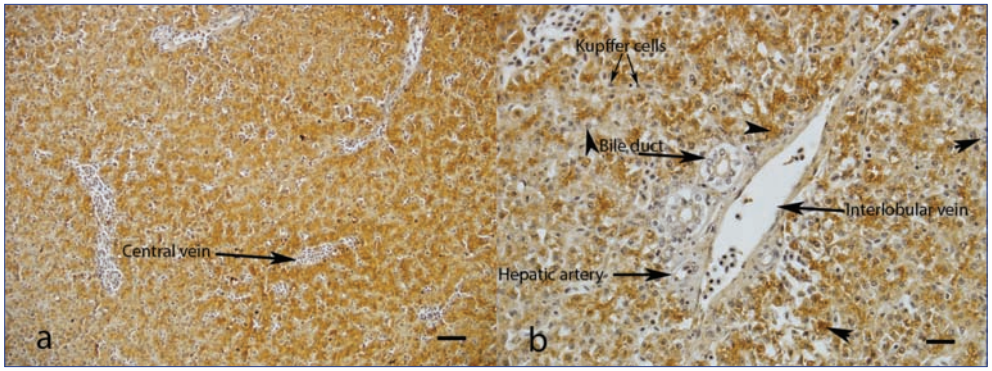

Fig 5. Catalase immunoreactivity in liver. **a-** Catalase immunoreactivity in liver, Bar: 100 µm; **b-** Catalase immunoreactivity in liver, *Arrow heads:* catalase immunoreactivity in hepatocyte in various degrees, Bar: 50 µm

liver. That finding is normal for tissues of avian species because lymphoid areas are seen as lymphoid follicles or lymphoid infiltration rather than lymphoid nodules [7,23,24]. The general histological features of the buzzard's liver and small intestine were basically similar to those of other avian species.

Kruidenier et al.[14] declared that catalase as an antioxidant enzyme in macrophages, and neutrophils in the lamina propria layer, prevented the harmful effects of H_2O_2 in the small intestine of mammalians. Dobashi et al.[25] reported that another antioxidant enzyme, glutathione peroxidase, had strong immunoreactivity in the lamina propria and weak immunoreactivity in the columnar cells, goblet cells and muscular layer in rats. The present study showed that catalase immunoreactivity was noticeable in the same parts of a buzzard's small intestine as those of rats. So, the present study's results were similar to those of Dobashi et al.[25]. Both these enzymes had a reaction in the same places in different species [14,25], which means that those parts of the intestines are important for antioxidant defence.

Morikawa and Harada [26] investigated immunohistochemical localization of catalase in mammalians and announced that this enzyme showed granular staining in the cytoplasm of the hepatocytes. In the same study, they reported that there was no reaction in the nuclei of hepatocytes. Bingol et al.[27] stated that there was diffuse cytoplasmic immunoreactivity and positive reaction in some nuclei in the hepatocytes of goose liver. The present study showed that catalase immunoreactivity was diffuse cytoplasmic in hepatocytes, in line with the results of Morikawa and Harada [26], and Bingol et al.[27]. As mentioned earlier, there was no immunoreactivity in the connective tissue, bile ducts and endothelial cells in the present study.

It was concluded that the general histological structure of the buzzard's small intestine and liver were similar to those in other avian species. Some areas in the small intestine and liver had catalase immunoreactivity, which means that an antioxidant defence occurs in those areas. The current study has provided information about the general structures of a buzzard's liver and small intestine, and it has also contributed to different studies about the antioxidant system in avians.

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Research Article

The mRNA Gene Expression Profiles for HSP60 and HSP70 in Various Aged Saanen Goats in Different Seasons [1]

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Abstract

In this study, the mRNA gene expression levels for HSP60 and HSP70 in various aged Saanen Goats in the spring were compared with the summer, fall and winter levels. The animal material was constituted by healthy 18 Saanen Goats at various ages. The animals were divided into three groups so that group I contained goats at 1-2 years old; group II 3-4 year- old-ones and group III 5-6 year-olds. In general, HSP60 and HSP70 mRNA expression levels were determined to have increased in all groups in the summer, fall and winter when compared with the levels in the spring which is known to be the most convenient season for goats in the region. The differences of mRNA expression levels for HSP60 and HSP70 among groups in the summer were found statistically insignificant. When the HSP60 expression levels in the winter in all groups were compared, the difference between Group I and the other two groups, Group II and III, was found statistically significant ($P<0.05$). The levels were seen to be higher in the young goats than in the old ones. This finding was that most significant aspect of this study. In other words, these levels decreased as the age increased. In resisting heat stres, to be able to define the roles and the functional mechanisms of mRNA gene expression levels of HSP60 and HSP70 in goats having high adaptation capacities to the environment particularly according to ages could be regarded as facilitators for the studies to be done on all species. In the following years, HSPs are believed to play significant roles in the selection of resistant animals particularly to the environmental conditions and to be one of the significant physiological parameters which will focus on farm animals.

Keywords: *Gene expression, mRNA, HSP 70, HSP 60, Saanen goats*

Farklı Yaşlardaki Saanen Keçilerinin Farkli Mevsimlerdeki HSP 60 ve HSP70 İçin mRNA Gen Ekspresyon Profilleri

Özet

Bu çalışmada, farklı yaşlardaki Saanen keçilerinin bahar mevsimine göre yaz, sonbahar ve kış mevsimlerindeki HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri karşılaştırılmıştır. Hayvan materyali olarak sağlıklı 18 baş Saanen keçisi kullanılmıştır. Hayvanlar yaşlarına göre üç gruba ayrılmıştır. Birinci grup hayvanlar; 1-2 yaşında, ikinci grup; 3-4 yaş, üçüncü grup hayvanlar; 5-6 yaşındadır. Genel olarak, bölgede keçiler için en uygun mevsim olan ilkbahardaki HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri yaz, sonbahar ve kış aylarında yaşlara göre karşılaştırılmıştır. Tüm gruplar için HSP60 ve HSP70 mRNA ekspresyon düzeyleri yaz için istatistiksel olarak önemsiz bulunmuştur. Kış mevsimde HSP60 mRNA gen ekspresyon düzeyleri gruplar arasında karşılaştırıldığında birinci grup ve diğer iki grup arasındaki fark istatistiki olarak önemli bulunmuştur ($P<0.05$). Gen ekspresyon düzeyleri genç olan birinci grupta, daha yaşlı olan diğer iki gruba göre daha yüksek bulunmuştur. Diğer bir değişle yaş artıkça ekspresyon düzeyi düşmektedir. Sıcaklık stresine karşı direnç bakımından adaptasyon yeteneği iyi olan keçilerde, özellikle yaşlara göre HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri, rolleri ve fonksiyonel mekanizmaları üzerine yapılacak çalışma sonuçları tüm türlerde yapılacak benzer çalışmalar için önemli olabilir. Gelecek yıllarda çevre koşullarına dirençli hayvanların seçiminde, HSP'lerin önemli rol oynayacağına inanılmakta ve çiftlik hayvanları için üzerinde odaklanılacak önemli bir fizyolojik parametre olacağı düşünülmektedir.

Anahtar sözcükler: *Gen Ekspresyonu, mRNA, HSP70, HSP60, Saanen keçileri*

INTRODUCTION

Heat stres, one of the environmental stresses, is the major constraint on animal productivity in tropical climatic conditions. Growth, productivity and reproductivity are impaired as a result of the drastic changes in biological functions caused by heat stress [1]. Determining the animal's thermotolerance ability is a must in order to

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measure the performance ability of a particular breed or animal against heat stress. Heat Shock Proteins (HSPs) are known as molecular chaperons and they maintain native structure of proteins and cell viability during stressful periods [2]. They are multigene families that range in molecular size from 10–150 kDa and are found in all major cellular compartments [3]. HSP60 is an important molecular chaperon under various stressful conditions [4]. These HSPs are known for their primary role as molecular chaperons that ensures the correct protein folding and apoptosis regulation during physiological stressful conditions. HSP60 is mostly found in the mitochondria. It helps in refolding of proteins and prevents aggregation of denatured proteins [3]. Another abundant and the best characterized HSP is the 70-kDafamily (HSP70) that consists of highly conserved stress proteins, expressed in response to stress, and plays crucial roles in environmental stress tolerance and adaptation [1]. HSP70 is mostly found in the cytosol and nucleus. Its functions are protein folding, cytoprotection, and as molecular chaperones [3].

Dairy breeds, especially the high-yielding animals, are typically more sensitive to heat stress as they generate more metabolic heat [5]. The heat stress effect becomes more detrimental when it is accompanied with high ambient humidity [6].

Goats adapted to a harsh environment perform better than other domesticated ruminants [7,8]. They are well adapted under different geographical and environmental conditions [1].

These animals have developed adaptive mechanisms that allow their survival at very high temperatures (45 to 50°C) as well as cold temperatures (–20 to –40°C). However, despite their extreme tolerance against temperature changes, the productivity of these animals softens the declines due to thermal stress [9]. Heat stress affects productivity of goats, but very little information is available about how they respond to heat at a cellular level. There is a strong correlation between the induction of HSPs and the induction of thermotolerance by preventing activation of stress kinases [10].

These proteins, called as Heat Shock Proteins (HSPs), have been determined to have increased not only due to high environmental or body temperatures but due to the stress factors, such as viral infections, heavy metals, chemical matterials, pesticides, oxygen and glucose deficiency as well [11,12].

Plenty of studies have recently been conducted on various diseases, particularly in humans, appearing with the aging due to dropping down HSP60 levels [13,14].

In this study, it was aimed to define the alterations in gene expression levels for HSP60 and HSP70 in various aged Saanen goat groups in different seasons. In resisting heat stress, to be able to define the roles and the functional mechanisms of mRNA gene expression levels of HSP60 and HSP70 in goats having high adaptation capacities to the environment particularly according to ages could be regarded as facilitators for the studies to be done on all species, including humans.

MATERIAL and METHODS

All experiments were done at the goat research unit in the Faculty of Agriculture and University of Adnan Menderes in AYDIN - Turkey whose location is (37° 45′ 03.31″ N and 27° 45′ 27.16″ E). It is 52 m above sea level. Experimental procedures were reviewed and approved by the Animal Ethics Committee of Adnan Menderes University, Aydin, Turkey (Approval No. B.30.2.ADU.2013/041). Mediterranean climate prevails in the region, namely summers are warm and dry and winters are rainy and warm. The annual average temperature is 17-18°C. Due to the north winds, the annual precipitation is between 580-1000 mm.

Animal Material

The animal material was constituted by healthy 18 Saanen Goats at various ages. The animals were divided into three groups. Group I contained at 1-2 years old goats; group II 3-4 year- old-ones and group III 5-6 year-olds. A constant and free feeding program containing energy and protein was applied throughout the year. For all of the goats in the groups, a constant and free feeding program in which the animals were free to reach their food freely 24 h in their pens or outside throughout the study period.

Recordings of Climatic Parameters

The experiments were carried out during four distinct phases coinciding with four seasons of the year, winter (mid-January), spring (mid -April), summer (mid-july) and autumn (mid-November). Blood sampling days were determined by tracking the meteorological agenda according to seasonal avareges for the spring and the falls and for the coldest day in the winter and the hottest day in the summer. All the animals were closely monitored and were provided similar managemental inputs during the experimental period.

The ambient temperature and humidity were continiously recorded all the year long with a hobo device installed a week before the first experiment day. Temperature Humidity Index (THI) was calculated to determine the effect of environmental conditions on animals. The following equation was used to calculate THI:

$$THI = T - (0.31-0.31*RH) * (T - 14.4)$$

where T is the dry bulb temperature (°C) and RH is the relative humidity (%) [6].

Blood Collection and RNA Extraction

On the experiment days, blood samples were taken from

the neck veins of the animals (vena jugularis) into lithium heparin-coated vacutainer tubes. As soon as the samples were taken, they were transported to the laboratory under refrigeration.

Total RNA was extracted from the blood with a commercial RNA isolation kit (High Pure RNA Isolation Kit, Roche, Version 12, 11828 665001) in accordance with the manufacturer's instructions. Supernatants were re-suspended by sterile DNase/RNase free water and the samples were stored at -80ºC until they were used. RNA concentrations were determined by optical density measurement at 260 and 280 nm. Purity was assesed by the 260/280 nm ratio.

Following RNA isolation, cDNA synthesis was performed using Light Cycler Nano Real Time PCR (Roche) for cDNA commercial kit (Transcriptor High Fidelity cDNA Synthese kit, Roche, Version 8, 05091284001).

Real time PCR analysis were performed using SYBR green (Roche, Fast Start Essential DNA Green Master), in acccordance with the manufacturer's procedure using a Light Cycler Nano Real Time PCR. All primers were synthesized by Genmar (İzmir/Turkey). The primer sequences as follows: HSP60-F 5'-ACTGGCTCCTCATCTCACTC -3'; HSP60-R 5'-TGTTCAATAATCACTGTCCTTCC-3', HSP70-F 5'-GACGAC GGCATCTTCAAG -3'; HSP70-R 5'-GACGACGGCATCTTCAAG -3', β actin-F 5'-AGTTCGCCATGGATGATGA-3'; β actin-R 5'-TGCCGGAGCCGTTGT-3'. β actin was also amplified in each assay as a control for using equal amounts of RNA in the RT-PCR reaction.

RT-PCR condition was an initial incubation at 40ºC for 10 minutes that was followed by a 10- minutes incubation at 95ºC then 45 cycles at 95ºC (10 s) , 60ºC (10 s) and 72ºC (15 s), and 20-second melting at 58ºC. The RT-PCR assay was repeated twice and the reproducibitiy was excellent with a correlation coefficient (r>0.95). The initial template was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction (Ct value). Relative gene expression was calculated using a $\Delta\Delta C_t$ method [15].

Statistical Analysis

Statistical analyses were preformed using the SPSS software v.22. The variables were investigated using Kolmogorov-Smirnov/Shapiro-Wilk's test to determine whether or not the parameters are normally distributed. As the measurements were not normally distributed the Kruskal-Wallis Analyse of Variance test were conducted to compare the parameters. The Mann-Whitney U test was performed to test the significance of pairwise differences. An overall 5% type-1 erroe level was used to infer statistical significance ($P<0.05$).

RESULTS

Climatic Data

The region where the study was conducted is under the effects of Mediterranean Climate. In other words, summers are dry and hot and winters are warm and rainy in the region. When the average values for the temperature and humidity data measured every single day of the year were evaluated, temperatures and THI values were seen to be the lowest in January and February and the highest in July and August.

According to the values given in *Table 1*, the average indoor temperature in the pen in July is 29.18ºC, and this value is in harmony with the long term average (28.4ºC for the last 10 years) for the province [16]. The daily temperature average measured in January was found as 8.63ºC and THI value as 9.09. On the other hand, the average values for the same parameteres in the summer were 29.18 and 26.67ºC respectively. The average temperatures in the spring and in the fall were found as 14.92 and 14.78ºC, respectively and the THI values were 14.26 and 14.74, respectively, all of which were close values to each other.

HSP60, HSP70 Gene Expression Levels

In this study, the spring season which has the best climatic comfort conditions for goats in this district was taken as the base. For this reason, the gene expression levels in the other seasons were compared with the values recorded in the spring.

When the mRNA gene expression levels for HSP60 in Group I (1 or 2 years old goats) were analized, the level measured in the winter was seen to be approximately 1.72 times more than the level measured in the spring. On the other hand, the difference between the other HSP60 expression levels for Group I determined in the summer and in the fall was found statistically insignificant. The

Table 1. *Temperature, Humidity and THI values belonging to experiment months which represent the four seasons lived in the region best*

Seasons	Month	Average Temperature (ºC)	Minimum Temperature (ºC)	Maximum Temperature (ºC)	Average Humidity (%)	THI
Winter	January	8.63	-6.12	22.52	74.2	9.09
Spring	April	14.92	2.94	29.85	57.57	14.26
Summer	July	29.18	18.61	40.53	46.00	26.67
Fall	November	14.78	5.96	25.12	62.08	14.74

expression level in the summer was found to be slightly more (1.2 times) than the spring level.

When the mRNA gene expression levels for HSP70 in Group I were examined, the level measured in the winter was seen to be approximately 1.68 times more than the level measured in the spring. The difference between the expression levels in the summer and in the fall was found statistically insignificant.

The seasonal differences among the mRNA gene expression levels for HSP60 in Group II (3 or 4 years old goats) were found statictically insignificant. The difference between the HSP60 expression levels in the summer and in the fall was found statistically insignificant.

When the HSP70 expression levels for Group II were analized, the level in the winter was seen to be 1.5 times more than the level in the spring.

The HSP60 and HSP70 expression levels in all seasons were compared in Group III and the differences were found statistically insignificant.

When the HSP60 expression levels in the winter in all groups were compared, the differences between Group I and the other two groups, Group II and III were found statistically significant. The difference between the HSP70 expression levels in Group I and II were found statistically insignificant. However, mRNA gene HSP70 expression level in Group I was approximately 1.25 times more than Group III, which was a significant finding.

When mRNA gene expression levels of the groups in

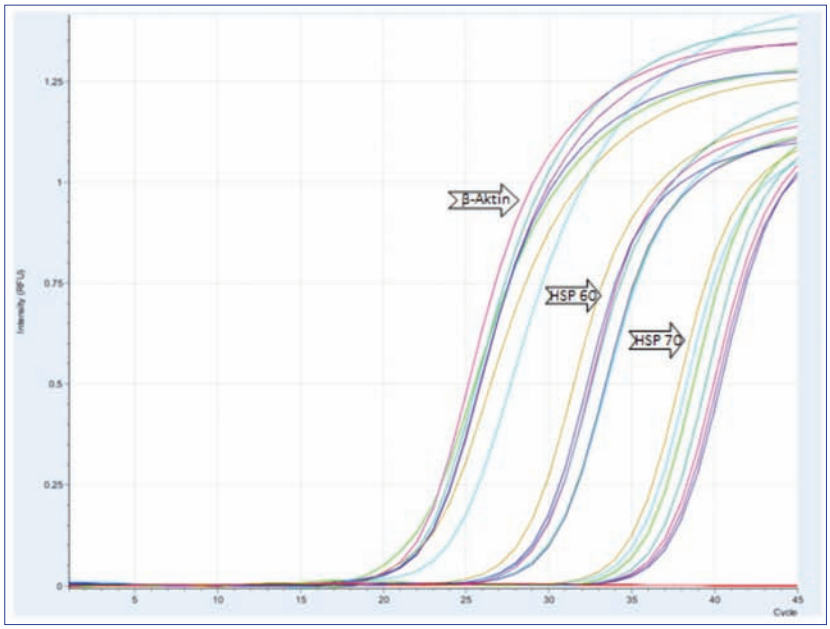
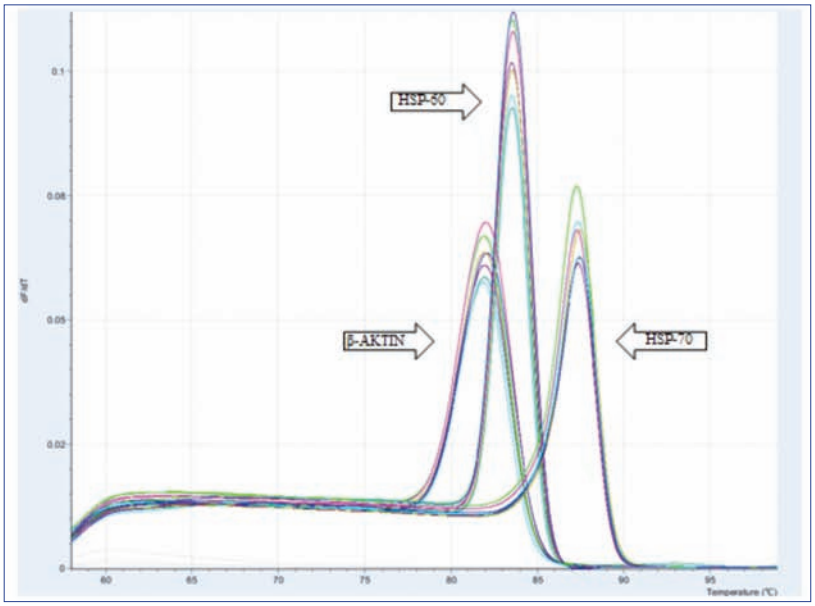


Fig 1. Synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR (The amplification curve of randomized spring blood sampling of the goats in Group II and III)

Fig 2. Melting point (Tm) graphics of β actin , HSP60, HSP70 mRNA expression



the spring for HSP60 were compared, Group I was seen to have 1.5 times higher expression levels than Group II and Group III, which was found statistically significant. The difference between Group II and Group III was found statistically non significant.

The differences of the mRNA genes HSP60 and HSP70 expression levels among the groups in the summer and in the fall were found statistically non-significant.

Fig. 1 is a Synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR (The amplification curve of randomized spring blood sampling of the goats in Group II and III).

Melting point (Tm) analysis was done in order to determine the purity of the samples expressed, whether there were interfering matters, whether the same gene region was analized in distinct samples and whether the primer dimer was created.

Fig. 2 contains Melting point (Tm) graphics of β actin, HSP60 and HSP70 mRNA expressions.

The synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR was shown in *Fig. 1*. As seen in *Fig. 2*, peakings at the same spot indicated that there were no interference and no primer dimer in the samples. The same case was valid for β actin as well which is used as reference gene and any primer dimer was the case in the reference gene as seen in *Fig. 2*.

DISCUSSION

In this study, HSP60 and HSP70 expression levels of various aged Saanen Goats in the spring were compared with the summer, fall and winter levels. In general, HSP60 and HSP70 mRNA expression levels were determined to have increased in all groups in the summer, fall and winter when compared with the levels in the spring which is known to be the most convenient season for goats in the region. With regard to the age groups, the greatest increase was recorded in the youngest group, Group I (1-2 years old). The values of the second age group, Group II (3-4 years old) were less than this increase and the lowest increase was measured for the oldest group, Group III (5-6 years old).

HSP60 and HSP70 expression levels were found higher in Group III than in the other groups in the winter. This increase in the winter in Group III, which contained older goats, was thought to be due to the pregnancy of the goats.

In this study, the differences of mRNA expression levels for HSP60 and HSP70 among groups in the summer were found statistically insignificant. In various studies comparing summer and winter values, the following results were determined. During summer seasons, HSP60 mRNA expression was found significantly ($P<0.05$) higher in all age groups in tropical and temperate region goats in comparison to winter season [3]. In a study, Increase of HSP70 gene expression during summer was observed to be higher in cold-adapted goat breeds [1].

In our study, the mRNA expression level for HSP60 in the fall was observed to have fallen as the age increased, and the the differences among the mRNA expression levels for HSP70in the three groups were found statistically non-significant.

The young, Group I, were observed to have expression levels three times more than the other groups and the levels decreased with aging. Similarly, the levels for HSP70 were high in the young group, and it also decreased with aging.

In a study they investigated HSP gene expression profiles belonging to goats in different seasons, ıt was found an increase in the mRNA gene expression level for HSP60 together with the age increase in the summer while they found the same levels in different age groups in winter statistically non-significant [3]. In a similar way, the HSP60 expression was indicated to have increased with aging in male Fischer 344xBrown Norway rats. Because of all these findings, it was concluded that apoptosis had increased and also the HSP60 increased, and for this reason, HSP60 increase could be possible with aging [17]. In a study he conducted, took blood samples from two different age groups (1-8 months old and 4-6 years old) of healthy Saanen goats subjected to the same management conditions. The mRNA expression levels for HSP60 and HSP70 was measured quantitatively using qRT-PCR SYBR Green Method. When the mRNA levels between the two groups were compared, the HSP60 expression levels were seen to be approximately 2 times lower in the old group than in the young group [18]. And similarly, the HSP70 expression levels were approximately 1.7 times lower, and it was observed that the older the goats, the lower HSP60 and HSP70 expression levels.

It was determined in various studies as well as in the ones conducted on humans that the mRNA expression levels for HSP60 and HSP70 decreased as the age increased. It was also determined that HSP60 and HSP70 levels diminished depending on the increase in the age in their study in which they searched the serum HSP60 and HSP70 levels in the individuals between 20 and 96 years old [19]. So they concluded that in the resisting ability against stres, there was a reduction related with the increasing age. Similarly, it was reported that mRNA expression levels for HSP70 in aged T lymphocytes [20]. It was found in a study they conducted on humans in various age groups that HSP70 level diminished depending on aging [21]. In another study, they conducted on young (5 months old) and old (24 months old) male Winstar rats that HSP70 expression was decreased in the shin and lung cells after a heat stres exposure [22]. That's why; they concluded that

HSP induction was disrupted with age. In a study they conducted on young (4-7 months old) and old (22-28 months old rats, in which they exposed all rats to 42.5°C for 30 min, found HSP70 expression in old rats 40-50% lower than young rats [23]. Holstein-Friesian milk cows was divided into 5 groups that Group 1 contained cows younger than 235 days old, Group 2 between 235-305 days old, Group 3 between 305-565 days old, Group 4 early lactation and Group 5 late lactation. HSP72 expression levels were found the highest in old cow milk group (305-565 days old). While HSP72 levels were low in late lactation group, they were found higher in early lactation group [24]. Similar studies in humans, it was found that HSP60 and HSP70 expression levels decreased with age and that a reduction happened in the ability to resist heat stress [19]. In another study, they conducted that HSP70 expression level was the best determiner with regard to the resistance to heat stress and that there was a logarhitmic relationship between HSP70 and resistance to heat stress in winged species. Due to their findings, they claimed that the HSP70 expression level in the cells was the indicator of the heat resistance power of the cells [25,26]. In a study, it was concluded that cyclical higher incubation temperatures at embryonic ages from 12 to 14 could increase liver HSP70 gene expression with no effect on body and liver weights, plasma T3, T4 and TG levels at day-old chicks [27]. In another study, it was demonstrated that the HSP70 expression significantly increased with the age in rainbow trout [28].

In this study, quantitative RT-PCR analyses of the HSP60 and HSP70 mRNA expressions according to seasons in various age groups of Saanen goats and gene expression amounts were compared among the groups composed that were defined numerically.

In conclusion, when the THI values were taken into account in the region where the study was conducted, severe heat stress was seen to have been effective in July and August, and heat stres was the case in June and July as well. In accordance with the other studies conducted, in the goats that were exposed to heat stress, mRNA expression levels for HSP60 and HSP70 were seen to be higher in the young goats than in the old ones. In other words, these levels decreased as the age increased. However, in the experiment done in the winter in this study, HSP60 value was seen to have increased as well although the age increased. It was thought that the pregnancy state of the goats could have been effective in these results, and HSP60 levels were found higher in the aged goats than younger ones.

In the study, HSPs were determined to be a significant mechanism against heat stress in humans like in farm animals. It is of great importance to breed resistant animals to particularly environmental conditions and to diseases and parasites on farms beside their productivity. Recently, researches on HSPs have focused on the development of cancer treatment and tumor vaccine in humans. In the following years, HSPs are believed to play significant roles in the selection of resistant animals particularly to the environmental conditions and to be one of the significant physiological parameters which will focus on farm animals.

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Research Article

Isolation and Antibiotic Susceptibilities of Beta Hemolytic *Streptococcus* Species from Various Body Site Infections with Cytologic Evidences in Thoroughbred and Arabian Racehorses in Turkey

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Abstract

Streptococcal microorganisms mostly beta hemolytic streptococci are the most important and widespread bacterial agents causing infectious diseases in horses and they often follow a difficult course. The goals of the present study are to determine; i) together with cytologic findings, the system distributions of the beta hemolytic streptococci as a primary pathogen, ii) the susceptibility patterns to the most commonly used antibiotics in thoroughbred and Arabian racing horses iii) the presence of multiple antibiotics resistance of beta hemolytic streptococci encountered in horses. Beta hemolytic streptococci isolated and cytologically neutrophilic inflammation confirmed samples which were submitted to Jockey Club of Turkey horse hospital laboratory between April 2014 - April 2017 were included into the study. The study was conducted on 128 samples including tracheal wash (71.9%), endometrial swabs (21.9%), abscess material (5.4%) and urine sample (0.8%). According to the isolation results, *S. equi* subsp. *zooepidemicus (S. zooepidemicus), S. dysgalactiae* subsp. *equisimilis (S. equisimilis)* and *S. equi* subsp. *equi (S. equi)* were isolated from whole samples as 80.4%, 14.1% and 5.5% respectively. Antibiotics susceptibility results demonstrated that amoxicillin/clavulanic acid (100%), amoxicillin (100%), ampicillin (100%), penicillin (100%), imipenem (100%), ampicillin/sulbactam (100%), rifampicin (97.6%), ceftiofur (100%) and ceftazidime (87.5%) were found to be effective, while enrofloxacin (14.8%), amikacin (0.8%), gentamicin (4.7%), kanamycin (0%), neomycin (0%), streptomycin (0%), oxytetracycline (7.0%) and trimethoprim/sulfamethoxazole (27.3%) were found to be mostly uneffective. The multiple antibiotics resistance rate was found to be 1.6% amongst beta hemolytic streptococci isolates in horses. These results of the present study confirmed the results of many similiar previous studies that *S. zooepidemicus* was the most frequently isolated beta hemolytic *Streptococcus* species followed by *S. equisimilis* and *S. equi.* In suspecious infections where a beta hemolytic streptococcal infection is suspected penicillin should be considered as the first line antimicrobial for horses when results of the samples submitted for bacterial culture and susceptibility test results are pending.

Keywords: *Antibiotic susceptibility, Beta hemolytic streptococci, Cytology, Horse, Lancefield group C Streptococcus spp*

Safkan İngiliz ve Arap Yarış Atlarında Görülen Beta Hemolitik *Streptococcus* Türlerinin Sitolojik Bulgular Eşliğinde Çeşitli Vücut Bölgelerinden İzolasyonu ve Antibiyotik Duyarlılıkları

Özet

Başta beta hemolitik türler olmak üzere streptokoklar atlarda bulaşıcı hastalıklara neden olan ve en sık görülen, en yaygın bakteriyel ajanlardır ve genellikle zor bir seyir izlemektedir. Bu çalışmanın amacı; i) sitolojik bulgularla beraber birincil patojen olarak beta hemolitik streptokokların atlardaki sistem dağılımlarını ii) safkan İngiliz ve Arap yarış atlarında en sık kullanılan antibiyotiklere karşı olan duyarlılıklarını iii) Türkiye' deki atlarda görülen beta hemolitik streptokokların çoklu antibiyotik dirençlilik oranlarını belirlemektir. Çalışmaya Nisan 2014 ile Nisan 2017 arasında Türkiye Jokey Kulübü İstanbul at hastanesi laboratuvarına gelen, beta hemolitik streptokok izole edilen ve sitolojik incelemede nötrofilik inflamasyon varlığı saptanan örnekler alınmıştır. Çalışmada %71.9'u trakeal aspirat sıvısı, %21.9'u endometriyal svap, %5.4'ü abse içeriği ve %0.8'i idrar örneği olmak üzere 128 örnek incelenmiştir. Çalışmada elde edilen sonuçlara göre tüm örneklerden *S. zooepidemicus, S. equisimilis* ve *S. equi* sırasıyla %80.4, %14.1, %5.5 oranlarında izole edilmiştir. Antibiyotik duyarlılık test sonuçlarına göre amoksisilin/klavulanik asit (%100), amoksisilin (%100), ampisilin (%100), penisilin (%100), imipenem (%100), ampisilin/sulbaktam (%100), rifamisin (%97.6), seftiofur (%100) ve seftazidim (%87.5) atlardan izole edilen beta hemolitik streptokoklara karşı duyarlı bulunurken, enrofloksasin (%14.8), amikasin (%0,8), gentamisin (%4.7), kanamisin (%0), neomisin (%0), streptomisin (%0), oksitetrasiklin (%7) ve trimetoprim sulfametoksazol (%27.3) çoğunlukla etkisiz bulunmuştur. Çoklu antibiyotik direnç oranı, izole edilen beta hemolitik streptokoklar arasında %1.6 olarak saptanmıştır. Bu çalışmadaki sonuçlar önceki benzer çoğu çalışmaların sonuçlarını tastik etmiş olup, *S. zooepidemicus* en sık izole edilen beta hemolitik streptokok türü olarak saptanmıştır. *S. zooepidemicus*'u, *S. equisimilis* ve *S. equi* izlemektedir. Şüpheli infeksiyonlarda kültür ve antibiyogram analizleri sonuçlanana kadar penisilin atlarda kullanılabilecek ilk antimikrobiyal olarak düşünülmelidir.

Anahtar sözcükler: *Antibiyotik duyarlılığı, At, Beta hemolitik streptokok, Lancefield grup C, Sitoloji, Streptococcus spp*

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INTRODUCTION

Beta hemolytic *Streptococcus* spp., which produce complete clearing of blood agar medium due to lysis of erythrocytes in media, are the most important and widespread agents causing infectious diseases in horses [1]. Of the beta hemolytic streptococci species *Streptococcus equi* subsp. *equi (S. equi), Streptococcus equi* subsp. *zooepidemicus (S. zooepidemicus)* and *Streptococcus dysgalactiae* subsp. *equisimilis (S. equisimilis)* and are three major beta hemolytic *Streptococcus* species, those cause severe and economically important diseases in horses [2,3].

S. equi is the causative agent of strangles, a highly contagious infection of the upper respiratory tract and associated lymph nodes of horses [2,3]. *S. equi* was also isolated from intra-uterine swabs (3.2%) [4], lower respiratory tract of foals (6.3%), adult horses (6.2%), lymph node abscess of foals (41.9%), adults (49.6%) and total isolation rate was found to be 6.3% in the same study [3]. *S. equi* was isolated 15.5% [1] and 12.8% [5] from various body sites of the horses in different studies.

S. zooepidemicus probably causes more disease in the horse than any other infectious agent yet [6]. *S. zooepidemicus* was the most isolated microorganism (72.0%) followed by *S. equisimilis* (21.3%), *S. equi* (5.8%) and unidentified beta hemolytic streptococci (0.9%) in a previous study and in different studies such as 56.4% [1] and 87.2% [5] amongst beta hemolytic streptococci. The agent was isolated 81.0% and 84.2% from respiratory tract of adult horses and foals respectively in the same study [3]. *S. zooepidemicus* is considered to be a mucosal commensal of the upper respiratory tract of horses which, causes disease as an opportunistic pathogen of the lower respiratory tract [2,3]. The agent is also responsible for the disease of reproductive tract especially when it causes endometritis in horses. Beta hemolytic *Streptococcus* was found to be the most frequently isolated microorganisms amongst other bacterial agents in equine uteri during fertility problems [7]. In equine genital tract, *S. zooepidemicus* was isolated 81.0% and 64.0% from adults and foals respectively [3]. The agent was isolated 67.8% from uterine swabs amongst beta hemolytic streptococci species [4]. It has also been reported as the most common bacterial agent causing placentitis in mares [8]. The agent was isolated from an aborted equine fetus in Turkey [9]. *S. zooepidemicus* was also reported as an etiologic agent in canine infectious respiratory disease [10] and has also been reported to cause septicaemia, meningitis, nefritis and arthritis in humans [11-14]. Molecular typing methods showed that human and equine isolates were identical and closely related, according to these datas *S. zooepidemicus* should also be recognized as an emerging zoonosis [15].

S. equisimilis is considered to be an infrequent pathogenic agent isolated from horses. It has been isolated from aborted placenta, less frequently from abscessed lymph nodes and from strangle-like disease of the upper respiratory system in horses [2,3,16]. The agent was also isolated from endometrial swabs (12.9%) [4], lower respiratory tract of the foals (12.4%) [3] and adult horses (12.4%) [3].

Besides the isolation of a microorganism from clinical samples, determining the inflammation by cytological examination in concordance with bacterial isolation is also detrimental in order to separate contamination from real infection in the diagnosis of bacterial infections [17,18].

An increased attention has been placed upon antimicrobial resistance in both medical and veterinary field [19]. *Streptococcus* group C agents isolated from endometrial swabs yielded 82.7% susceptibility to amoxicillin/clavulanic acid, 67.7% ampicillin, 8.4% enrofloxacin, 6.7% gentamicin, 65.2% penicillin, 51.3% rifampicin, 17.0% trimethoprim/sulfamethoxazole and 15.1% tetracycline in a study performed in Italy [7]. Ampicillin, gentamicin, neomycin, oxytetracycline, penicillin G, trimethoprim/sulfamethoxazole yielded 100%, 19%, 13%, 29%, 100% and 90% efficacy against beta hemolytic streptococcal isolates in a different study respectively [4]. Penicillin showed 100% efficacy against *S. equisimilis*, *S. equi* and *S. zooepidemicus* [1]. The efficacy of streptomycin were determined as 33.3%, 0%, and 10.0%, neomycin 50.0%, 0% and 23.5%, gentamicin 100%, 100% and 97.4%, tetracycline 47.1%, 60.0% and 60.0%, amoxicillin/clavulanic 100% against the three pathogens, while the efficacy of enrofloxacin was found to be 73.3%, 81.8% and 86.8% against *S. equisimilis*, *S. equi* and *S. zooepidemicus* respectively in the same study [1]. Erol et al.[3] reported that gentamicin efficacy was 83.3%, 82.8% and 87.5% against *S. equi*, *S. equisimilis* and *S. zooepidemicus* respectively where penicillin efficacy was found to be 98.7%, 99.2%, 98.9%, tetracycline efficacy was found to be 98.8%, 50.9%, 44.0% and trimethoprim/sulfamethoxazole efficacy was found to be 50.2%, 94.4%, 30.9% against the same agents respectively. Results from these studies enlight practitioners in making an evidence based antimicrobial medicine choice where rapid treatment is needed and culture-susceptibility results cannot be awaited. Bacterial resistance profiles can vary over time. Thus continuous local surveillance has great importance [20].

There has been also a great concern of multidrug resistant bacteria in veterinary field. Multidrug resistance was defined as resistance to three or more antimicrobial classes in pathogens isolated from animals [19]. In New Zealand, multidrug resistance rate of *Streptococcus* spp. was found to be 3.9% and age of the horse was determined to be significantly associated with multidrug resistance statistically [19].

The goals of the present study are to determine; i) together with cytologic findings, the system distributions of the beta hemolytic streptococci as a primary pathogen, ii) the susceptibility to the most commonly used antibiotics in

thoroughbred and Arabian racing horses iii) the presence of multiple antibiotics resistance of beta hemolytic streptococci encountered in horses.

MATERIAL and METHODS

Samples

Only beta hemolytic streptococci isolated and cytologically neutrophilic inflammation confirmed samples were included into the study. The study was conducted on 128 samples. All the samples were submitted between April 2014 and April 2017 into the Jockey Club of Turkey Istanbul Veliefendi Racetrack Horse Hospital. The samples, from which beta hemolytic streptococci isolated, were consisted of 71.9% (n=92) tracheal wash, 21.9% (n=28) endometrial swabs, 5.4% (n=7) abscess material and %0.8 (n=1) urine sample. Nasal swabs were not included to the study due to high content of beta hemolytic streptococci and other bacterial agents as normal flora of upper airways in horses [2,16]. Tracheal wash samples were collected according to the previous study [18]. Briefly, 40 mL sterile physiologic saline solution was instilled from proximal trachea and immediately aspirated back to a 60 mL sterile injector with a flushing catheter elongated via the biopsy channel of the endoscopy. Maximum precautions were taken to reduce the risk of upper airway contamination. After collecting the samples, they were transferred to a plane tube for bacteriology and EDTA tube for cytologic examination. Endometrial swabs were collected according to the previous study [21]. Briefly double guarded swabs were used to collect the samples. The instrument was protected with the gloved hand of the veterinarian when introduced in the vagina and directed to the cervix. The inner sheath of the instrument was advanced through the cervix and the swab was rolled several times against the endometrium. Approximately 30 sec later the swab was retracted in the inner sheath while the outer one was left in the place within the cervix for the second sampling. Abscess materials were collected with sterile needles into sterile 5 mL syringes and they were immediately sent to the laboratory for culture. Horses' age information were also recorded. 0-2 years old animals were classified as foals. Horses older than 3 years old≤ were classified as adult horses [3].

Cytology

Slide preparations of the tracheal wash samples were made by centrifugation of the samples at 1500 rpm. for 5 min. The slides were prepared from the sediment of the centrifuged samples as described before [17]. The air dried slides were stained with May Grunwald Quick stain (Bio Optica 20134 Milano Italy) that was used according to the manufacturer's instructions. The slides were examined under 100x magnification microscope and percentage (%) of the neutrophils, macrophages, lymphocytes, eosinophils and mast cells were determined by counting 300 cells [18]. Inflammation of the lower airways was considered positive when neutrophils made up ≥30% of all cells and the presence of degeneration in neutrophils and likely to have intracellular bacteria *(Fig. 1)* [17]. Cytological examination of the intrauterine swabs were performed according to the previous study [22]. Endometrial swabs were smeared on a microscope glass slide after streaking to the appropirate agars for culture. The slides were then stained using May Grunwald Giemsa stain (Bio Optica 20134 Milano Italy). All slides were examined under the microscope (40x magnification) for the presence of polimorphonuclear cells (PMNs). The sample was considered as positive for inflammation when PMNs made up ≥0.5% of all cells. Cytologic examination of the urine samples were performed by centrifuging the urine at 3.000 rpm for 5 min. After the centrifuge, the supernatant was discarded and sediment was examined microscopically using high dry objective (40X). The sample was accepted as positive, if white blood cells are observed 3< high power field [23].

Bacteriology and Antibiotics Susceptibility Testing

Isolation of beta hemolytic streptococci from tracheal wash samples, intrauterine swabs and abscess materials were carried out as described before [5,24]. Briefly, the samples were inoculated in 5% sheep blood agar in both aerobic and microaerobic (5% CO_2) conditions at 37°C. The media were incubated 24-48 h for beta hemolytic streptococci growth.

The criteria used for reporting a culture for beta hemolytic

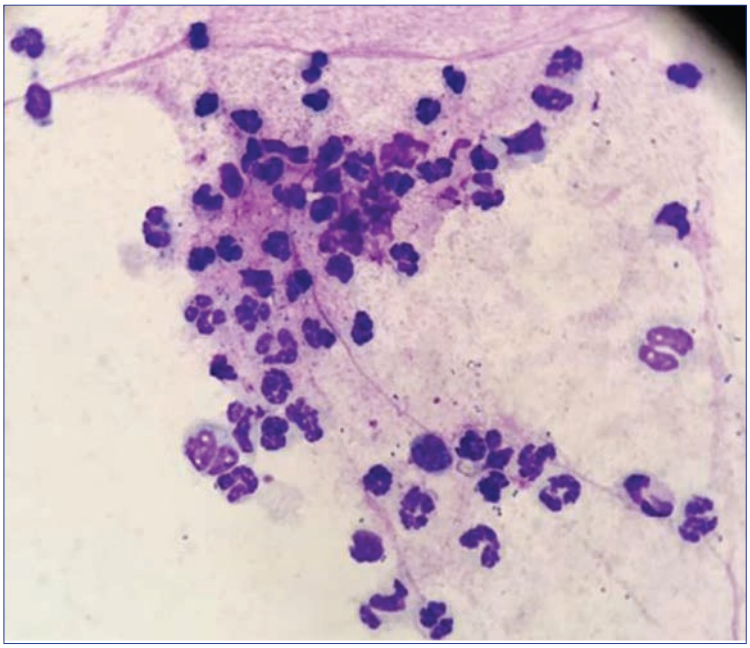

Fig 1. Septic neutrophilic inflammation in a tracheal wash sample (100x magnification)

streptococci growth was the isolation of the micro-organisms in pure culture or predominating on the agar plate as previously described [8].

Identification of the suspected colonies were carried out by subjecting them to Gram stain (GBL, İstanbul), catalase test, latex agglutination test (Oxoid Ltd., Basingstoke, UK) to determine serological group and then finally determining their biochemical profile using API 20 Strep ID kit according to the manufacturer's instructions (bioMerieux S.A., Marcy l'Etoile, France) respectively.

In vitro antibiotics susceptibility testings were performed by using disc diffusion method (25) according to the standards of the Clinical Laboratory Standards Institute (CLSI). Isolates were reported as susceptible to an antimicrobial if the diameter of the zone of inhibition was greater than the breakpoint for that drug, according to the CLSI Standards [26]. Amikacin (30 µg), amoxicillin/clavulanic acid (30 µg), amoxicillin (25 µg), ampicillin (10 µg), ceftiofur (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), kanamycin (30 µg), neomycin (10 µg), oxytetracycline (30 µg), penicillin (10 IU), streptomycin (10 µg), ampicillin sulbactam (20 µg), trimethoprim/sulfamethoxazole (25 µg) and ceftazidime (30 µg) antimicrobial discs (Oxoid Ltd., Basingstoke, UK) were included in the susceptibility tests. Multidrug antimicrobial resistance was also assessed which was described as an isolate being resistant to three or more of the following antimicrobial agents: enrofloxacin, gentamicin, ceftiofur, penicillin, oxytetracycline and trimethoprim-sulfonamide combination [19].

Statistical Analysis

The statistical analysis of the association between age groups (including ≤2 years old and 3≤ years old) and the amount multidrug resistant isolates were evaluated using the Pearson Chi-Square (X^2) test with Statistical Package for Social Sciences (SPSS) [27].

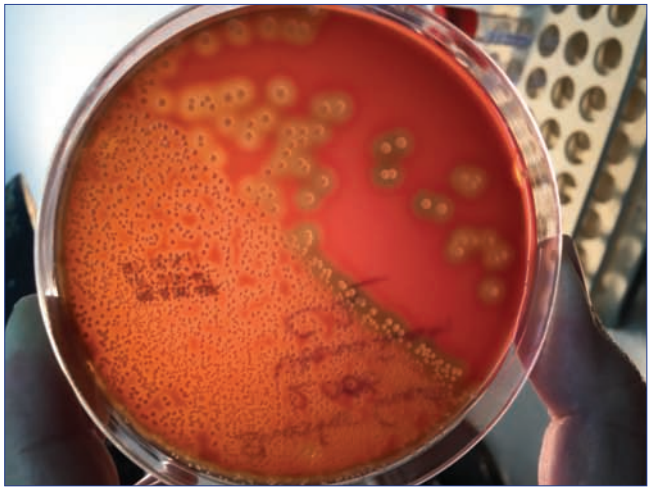

Fig 2. Beta hemolytic Streptococci colonies on 5% sheep blood agar. The isolated colonies in the picture were identified *as Streptococcus equi* subsp. *equi*

RESULTS

Beta hemolytic streptococci isolated from 128 samples including tracheal washes, endometrial swabs, abscess material and urine samples were *S. equi* (5.5%) *(Fig. 2)*, *S. equisimilis* (14.1%) *(Fig. 3)* and *S. zooepidemicus* (80.4%) *(Fig. 4)*, which were identified according to their biochemical profile.

When the sites of isolations were examined, *S. zooepidemicus* (88.0%), *S. equisimilis* (10.9%) and *S. equi* (1.1%) were

Fig 3. *Streptococcus dysgalactiae* subsp. *equisimilis* colonies isolated from endometrial swab

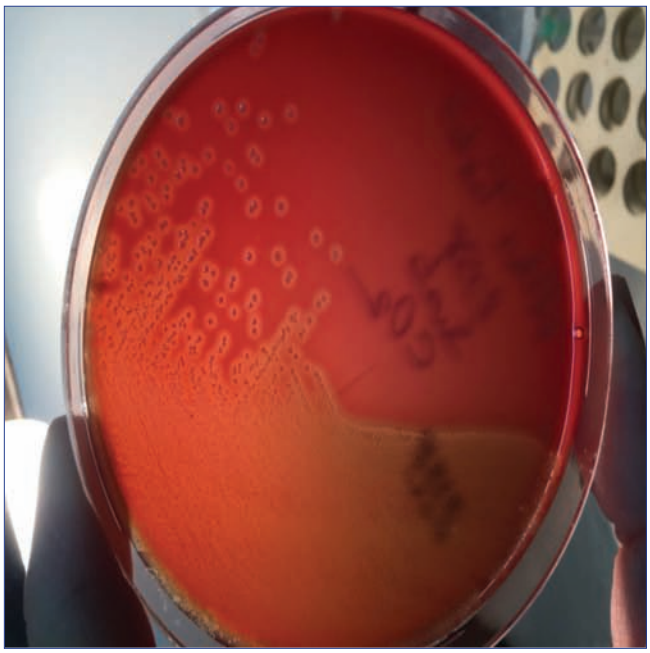

Fig 4. *Streptococcus equi* subsp. *zooepidemicus* colonies isolated from endometrial swab

Table 1. *Distribution of recovered microorganisms according to the sample type*

Isolated Microoganisms	Tracheal Wash (n=92)	Endometrial Swab (n= 28)	Abscess Material (n=7)	Urine Sample (n=1)
S. equi subsp. *zooepidemicus*	81 (88.0%)	16 (57.1%)	5 (71.4%)	1 (100%)
S. dysgalactiae subsp. *equisimilis*	10 (10.9%)	7 (25.0%)	1 (14.3%)	0 (0%)
S. equi subsp. *equi*	1 (1.1%)	5 (17.9%)	1 (14.3%)	0 (0%)

Table 2. *Distribution of isolated microorganisms according to the isolation year*

Isolated Microorganisms	2014 (April-December) (n= 39)	2015 (n=53)	2016 (n=25)	2017 (January-April) (n=11)
S. equi subsp. *zooepidemicus*	33 (84.6%)	50 (94.3%)	17 (68.0%)	3 (27.3%)
S. dysgalactiae subsp. *equisimilis*	5 (12.8%)	2 (3.8%)	5 (20.0%)	7 (63.6%)
S. equi subsp. *equi*	1 (2.6%)	1 (2.6%)	3 (12.0%)	1 (9.1%)

Table 3. *Distribution of isolated microorganisms according to the age groups*

Isolated Microorganisms	Foals (0-2 age) (n=51)	Adult Horses (3≤age) (n=77)
S. equi subsp. *zooepidemicus*	43 (84.3%)	60 (77.9%)
S. dysgalactiae subsp. *equisimilis*	7 (13.7%)	11 (14.3%)
S. equi subsp. *equi*	1 (2.0%)	6 (7.8%)

Table 4. *Antibiotic susceptibilities of the isolated microorganisms*

Antimicrobials	Beta Hemolytic Streptococci (n=128)	*S. equi* subsp. *zooepidemicus* (n=103)	*S. dysgalactiae* subsp. *equisimilis* (n=18)	*S. equi* subsp. *equi* (n=7)
Amoxycillin/Clavulonic acid	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Amoxycillin	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Ampicillin	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Penicillin G	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Imipenem	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Ampicillin/Sulbactam	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Enrofloxacin	19 (14.8%)	18 (17.5%)	1 (5.5%)	0 (0%)
Rifampicin	125 (97.6%)	100 (97.1%)	18 (100%)	7 (100%)
Ceftiofur	128 (100%)	103 (100%)	18 (100%)	7 (100%)
Ceftazidime	112 (87.5%)	90 (87.4 %)	16 (88.9%)	6 (85.7%)
Amikacin	1 (0.8%)	1 (0.9%)	0 (0%)	0 (0%)
Gentamicin	6 (4.7%)	6 (5.8%)	0 (0%)	0 (0%)
Kanamycin	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Neomycin	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Streptomycin	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Trimethoprim/Sulfamethoxazole	35 (27.3%)	23 (22.3%)	8 (44.4%)	4 (57.1%)
Oxytetracycline	9 (7.0%)	6 (5.8%)	2 (11.1%)	1 (14.3%)

isolated from 92 tracheal wash samples. While examining 28 endometrial swabs *S. zooepidemicus* (57.1%), *S. equisimilis* (25.0%) and *S. equi* (17.9%) were isolated respectively. From 7 abscess materials *S. zooepidemicus* (71.4 %), *S. equisimilis* (14.3%) and *S. equi* (14.3%) were isolated. From 1 urine sample *S. zooepidemicus* (100%) was isolated *(Table 1)*.

Distribution of the isolated beta hemolytic streptococci according to the isolation years revealed that *S. zooepidemicus* was the most isolated pathogen in 2014, 2015, 2016. *S. zooepidemicus* was followed by *S. equisimilis* and *S. equi* respectively. *S. equisilimis* seemed to be the most isolated microorganism between January 2017

and April 2017 to the contrary of 2014, 2015 and 2016 *(Table 2)*.

When examining horse age groups in terms of beta hemolytic streptococci isolation, it has been shown that isolation rate of *S. zooepidemicus* was 84.3% in young horses/foals (0-2 years old) while the isolation rate of *S. equisimilis* and *S. equi* were 13.7% and 2.0% respectively. In adult horse group (2 years old<) *S. zooepidemicus*, *S. equisimilis* and *S. equi* were isolated 77.9%, 14.3% and 7.8% respectively *(Table 3)*.

The present study's antibiotics susceptibility test results showed that all beta hemolytic streptococci agents were susceptible to amoxicillin/clavulanic acid, amoxicillin, ampicillin, penicillin, imipenem, ampicillin/sulbactam and ceftiofur antibiotics. They were resistant to kanamycin, streptomycin and neomycin *(Table 4)*.

When the recovered microorganisms were examined individually *S. zooepidemicus*, *S. equisimilis* and *S. equi* isolates were all susceptible to amoxicillin/clavulanic acid, amoxicillin, ampicillin, penicillin G, imipenem, ampicillin/sulbactam and ceftiofur antibiotics, *S. equisimilis* and *S. equi* isolates were all also susceptible to rifampicin. All of the isolates of *S. zooepidemicus*, *S. equisimilis* and *S. equi* were resistant to kanamycin, neomycin and streptomycin. Additionally all *S. equisimilis* and *S. equi* isolates were resistant to amikacin and gentamicin *(Table 2)*. In the present study multi drug resistant isolates were cultured from 2 (1.6%) samples. Both of these isolates were resistant to gentamicin, oxytetracycline and trimethoprim/sulfamethoxazole.

DISCUSSION

Beta hemolytic streptococci species were known to be the primary bacterial pathogens of the horse. In the present study *S. zooepidemicus*, *S. equisimilis* and *S. equi* were isolated as 80.4%, 14.1% and 5.5% from clinical samples respectively. Neither, unidentified beta hemolytic streptococci nor, any other beta hemolytic streptococci species were isolated. *S. zooepidemicus*, *S. equisimilis*, *S. equi* and unidentified beta hemolytic streptococci were isolated as 72.0%, 21.3%, 5.8% and 0.9% in the same study respectively [3]. A different study determined the isolation rates of *S. zooepidemicus* 56.4%, *S. equisimilis* 23.9%, *S. equi* 15.5% and *S. agalactiae* 4.2% from a wide range of sample types including tracheal swabs, urine [1]. These results showed that the isolation rates of these agents were similar to the present study and *S. zooepidemicus* was the most isolated pathogenic agent amongst beta hemolytic streptococci in all studies. *S. equisimilis* was the second most isolated agent and *S. equi* was the third most isolated one in the present study, which was similar to the previous studies [1,3].

Analysing the results according to age groups revealed that *S. zooepidemicus*, *S. equisimilis* and *S. equi* were isolated 84.3%, 13.7% and 2.0% from the foals, 77.9%, 14.3% and 7.8% from the adult horses respectively in the present study. In a previous study, *S. zooepidemicus*, *S. equisimilis* and *S. equi* were isolated 77.5%, 11.5% and 10.4% from foals, 74.1%, 18.1% and 6.9% from adult horses respectively [3]. Despite isolation rate differences between two studies, which might had been due to the geographic differences and population difference, both of these studies suggested that *S. zooepidemicus* was the most frequently isolated agent both in foals and adult horses followed by *S. equisimilis* and *S. equi* respectively.

In a study examining the mares with fertility problems, a total of 31 beta hemolytic streptococci consisted of 67.8% *S. zooepidemicus*, 12.9% *S. equisimilis*, 3.2% *S. equi* and 16.1% unidentified agents were isolated from endometrial swabs [4]. In the present study *S. zooepidemicus*, *S. equisimilis* and *S. equi* were isolated 57.1%, 25.0% and 17.9% from 28 endometrial swabs. There were differences in the isolation rates of the agents but it could be concluded that *S. zooepidemicus* was the most frequently isolated agent followed by *S. equisimilis* and *S. equi* amongst beta hemolytic streptococcal agents from intrauterin infections of mares in both studies. Additionally *S. zooepidemicus* was also isolated from an urine sample with the presence of leukocytes that supports the presence of inflammation. From all these datas, it could be concluded that beta hemolytic streptococcal agents should not be ignored in horses' uro-genital infections besides of other microbial agents.

In the present study *S. zooepidemicus* (88.0%) is the most frequently isolated agent from respiratory tract samples in both foals and adult horses. This result is in agreement with results of a retrospective study performed in U.S. [3]. The reason of high isolation rate of *S. zooepidemicus* might had been due to being a normal mucosal flora bacteria of the upper respiratory tract in healthy horses and for any reason that caused immuno compromisation such as stress, transportation, racing or anatomical predisposition could let the agent migrate to lower respiratory tract and finally might had caused infection [2]. According to the present study, *S. equisimilis* (10.9%) is the second most frequently isolated agent followed by *S. equi* (1.1%) which showed agreement with the other retrospective study [3].

Beta hemolytic streptococci species were found to be 100% susceptible against amoxycillin/clavulanic acid, amoxycillin, ampicillin, penicillin, imipenem, ampicillin/sulbactam and ceftiofur according to the present study in vitro. These results are mostly in agreement with previous studies [1,3-5,19].

Ceftazidime and rifampicin were also effective against beta hemolytic streptococci species that were isolated in the study (87.5% and 97.6% respectively). Unfortunately no work at the literature was found that use disc diffusion method to compare the suspectibility testing results of

the present study for ceftazidime in horses. Efficiency of rifampicin against *Streptococcus* spp. group C was found to be 51.3% in endometrial swabs [7]. However, in the present study the efficiency of rifampicin against the same microbials was found as 96.4% in endometrial swabs. The variations between the results of these two studies could be due to the frequency of the use of rifampicin in two different countries/regions.

Aminoglycoside group antimicrobials (amikacin, gentamicin, kanamycin, neomycin, streptomycin) showed very low or zero efficacy against beta hemolytic streptococci species in the present study. These results are mostly in agreement with the previous studies [1,3,5,77,19] except gentamicin. Gentamicin showed high antimicrobial efficacies such as 84.5% [3], 99.1% [1], 90.0% [5] against beta hemolytic *Streptococcus* spp. and 91.6% against *Streptococcus* spp. in previous studies. But on the other hand some studies demonstrated low efficacies such as 19% [4] and 6.7% [7]. In the present study efficacy of gentamicin was found 4.7% which is similar with low efficacy determined studies. Variations in the efficacy rate of gentamicin might had been due to the wide spread empirical use of gentamicin in clinical practice.

Trimethoprim/sulfamethoxazole and enrofloxacin antimicrobial efficacy results of the present study revealed lower efficacy rates such as 27.3%, 22.3%, 44.4%, 57.1% for trimethoprim/sulfamethoxazole and 14.8%, 17.5%, 5.5%, 0% for enrofloxacin against beta hemolytic streptococci, *S. zooepidemicus*, *S. equisimilis* and *S. equi* respectively than previous studies [1,3,5,19]. But higher efficacy rates for sulphamethaxzole/trimethoprim and similar efficacy rates for enrofloxacin were also obtained when comparing the present study with a previous one [7].

The susceptibility results of oxytetracycline showed an efficacy of 7.0%, 5.8%, 11.1% and 14.3% against beta hemolytic *Streptococcus* spp., *S. zooepidemicus*, *S. equisimilis* and *S. equi* respectively in the present study. However in two different studies, the efficacy of tetracycline was found to be higher than the present study such as 64.6%, 44.0%, 50.9%, 98.8% and 55.7%, 60%, 47.1%, 60.0% against beta hemolytic *Streptococcus* spp., *S. zooepidemicus*, *S. equisimilis* and *S. equi* respectively [1,3]. On the contrary in a different study performed in Italy showed *Streptococcus* group C microorganisms showed low susceptibility (15.1%) against tetracycline [7]. From all these results it could be concluded that efficacy rate of oxytetracycline could change between countries/regions due to the frequency of use in clinical practice and local demonstration of oxytetracycline efficacy like other antimicrobials against beta hemolytic streptococci has paramount importance.

Based upon the antimicrobial susceptibility results of the present study, multidrug resistance was found 1.6% (2/128) in beta hemolytic streptococci isolates and no significant association could be found for the presence of the multidrug resistant bacteria in different age groups (≤2 years old and ≥3 years old). In New Zealand multidrug resistance was found to be 3.9% (12/310) amongst streptococci isolates and a significant association was found for the presence of multidrug resistant bacteria in 2 years old horse group [19]. More extensive studies including high numbers of horses and isolates should be set in order to deeply highlight multidrug resistance status of bacterial agents in Turkey.

In conclusion, the current study yielded that *S. zooepidemicus* was found to be the most frequently isolated beta hemolytic streptococci in Turkey. *S. zooepidemicus* has also zoonotic significance so that care must be taken by the practitioners and related persons to horses while handling suspected or confirmed cases with this microbial agent. Beta hemolytic *Streptococcus* spp. demonstrated high susceptibility to amoxycillin/clavulanic acid, amoxycillin, ampicillin, penicillin, imipenem, ampicillin/sulbactam, ceftiofur, rifampicin and ceftazidime while showing low susceptibility or resistance to enrofloxacin, amikacin, gentamicin, kanamycin, streptomycin, neomycin, oxytetracycline and trimethoprim/sulphamethaxzole in the present study. It can also be concluded that penicillin can be considered as a first line antimicrobial for use in horses in Turkey where a beta hemolytic streptococcal infection is suspected, when results of the samples submitted for bacterial culture and susceptibility testing are pending. No significant association was found between age groups (≤2 years old and ≥3 years old) and isolation of multidrug resistant beta hemolytic streptococci.

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Research Article

Molecular Characteristics of *Pasteurella multocida* Strains Isolated from Poultry in China and Genetic Analysis of Strains in Terms of the *tonB* Gene

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Abstract

Pasteurella multocida (P. multocida) causes fowl cholera in birds and the pathogenesis and virulence factors involved are still poorly understood. TonB protein is a periplasmic protein prevalent in a large number of Gram-negative bacteria, as in Pasteurella, which is believed to be responsible for the import of ferric iron complexes across the outer membrane and plays a role as an important virulence factor to help bacteria to obtain nutrients. In this study, a total of 23 isolates of *P. multocida* were obtained from 172 cases of clinical respiratory disease in duck and geese, giving an isolation rate of 13.4%. *P. multocida* Serogroup A was isolated from 22 cases (95.7%), whereas serogroup F was from 1 case (4.3%). All isolates were analyzed for their susceptibility to 15 antibiotics and the presence of 18 genes for virulence factors. The susceptibility profiles suggested that Tetracycline, Cephalosporin, Enrofloxacin, and Aminoglycosides were the drugs most likely to be active against *P. multocida*. However, 78.3% and 52.2% of poultry strains were resistant to Penicillin and Sulfisoxazole, respectively. PCR results showed that *ptfA, fimA, tonB, fur, hgbA, hgbB, sodA, sodC, pmHAS, nanH, nanB, plpB* and *ompH* genes occur in most poultry strains of *P. multocida*. The virulence genes such as *toxA, tadD, hsf-1, pfhA* and *ompA* were each present in <73.9% of strains. The *tonB* gene was detected in all 23 clinical strains of *P. multocida* which were recovered from different hosts and harboring different serotypes. Phylogenetic analysis indicated that these *tonB* genes of *P. multocida* from different hosts and harboring different serotypes have close genetic relationship with a high similarity. Multiple sequence alignment demonstrated that TonB protein of Gram-negative bacteria exists in multiple conserved sites, such as -xSSGx-, -YP-, -LD- and xA[A/V]Lx motif. These findings provide clinical data into the epidemiological and molecular characteristics of avian *P. multocida* isolates and provide a reference for the researches of drug target in *P. multocida*.

Keywords: *Antimicrobial susceptibility, Capsular antigens, Pasteurella multocida, tonB gene, Virulence gene, Cloning, Phylogenetic analysis*

Çin'de Kanatlı Hayvanlardan İzole Edilen *Pasteurella multocida* Sışlarının Moleküler Karakterizasyonu ve *tonB* Geni Açısından Genetik Analizi

Özet

Pasteurella multocida (P. multocida) kuşlarda kanatlı kolerasına neden olur ve hastalıkta virulans faktörleri hala tam olarak anlaşılamamıştır. TonB proteini *Pasteurella* da dahil pek çok Gram-negatif bakteride yaygın olarak bulunan periplazmik bir protein olup membran dışına ferrik demir komplekslerini taşımakta görevlidir ve bakterilerde besin sağlamaya yardım etmek suretiyle önemli bir virulans faktörü olarak rol oynar. Bu çalışmada, 172 klinik solunum hastalıklı ördek ve kazdan toplam 23 *P. multocida* izolatı elde edilerek %13.4'lük izolasyon oranı sağlandı. *P. multocida* Serogrup A 22 vakadan izole edilirken (%95.7), serogrup F 1 vakadan izole edildi (%4.3). Tüm izolatlar 15 antibiyotiğe karşı duyarlılıklarına ve virulans faktör olarak 18 genin mevcudiyetine göre analiz edildi. Duyarlılık profili, *P. multocida*'nın tetrasiklin, sefalosporin, enrofloksasin ve aminoglikozidlere karşı duyarlı olduğunu gösterdi. Ancak, elde edilen suşların %78.3'ü penisiline ve %52.2'si sulfisoksazola karşı dayanaklıydı. PCR sonuçları çoğu kanatlı *P. multocida* suşlarında *ptfA, fimA, tonB, fur, hgbA, hgbB, sodA, sodC, pmHAS, nanH, nanB, plpB* ve *ompH* genlerinin olduğunu gösterdi. *toxA, tadD, hsf-1, pfhA* ve *ompA* gibi virulans genlerinin her biri suşların <73.9%'da mevcuttu. *tonB* geni *P. multocida*'nın tüm 23 klinik suşlarında farklı konakçılardan elde edildi ve farklı serotipleri içermekteydi. Filogenetik analiz sonucunda farklı konakçılardan elde edilen ve farklı serotipleri içeren *P. multocida tonB* genlerinin yakın genetik akrabalıkları olduğunu ve yüksek oranda benzerlik gösterdiği belirlendi. Multiple sekans hizalama ile Gram-negatif bakterilerin TonB proteinin -xSSGx-, -YP-, -LD- ve xA[A/V]Lx motif gibi çok sayıda korunmuş bölgesinin olduğu gösterildi. Elde edilen bulgular avian *P. multocida*'nın epidemiyolojik ve moleküler karakterizasyonu için klinik veri ile *P. multocida*'ya karşı ilaç üretiminde araştırmacılar için bir referans sağlamıştır.

Anahtar sözcükler: *Antimikrobiyal duyarlılık, Kapsül antijenleri, Pasteurella multocida, tonB geni, Virulans geni, klonlama, Filogenetik analiz*

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INTRODUCTION

Pasteurella multocida (P. multocida) is a Gram-negative pathogen causing severe zoonosis and it is widely believed that this bacterium through tissues of the respiratory and digestive tracts results in the occurrence of pasteurellosis [1,2]. *P. multocida* is associated with the several distinct diseases. These can be classified into the two general groups including hemorrhagic septicemia and respiratory system diseases, for instance, avian cholera, hemorrhagic septicemia in ungulates, atrophic rhinitis in pigs and snuffles in rabbits [3]. *P. multocida* strains are classified into the serogroups (A, B, D, E and F) based on the capsule antigens, and there is a significant association between various capsular serotypes and pathogenicity [4]. The pathogenicity of *P. multocida* is associated with the different virulence factors. A number of virulence factors identified of *P. multocida* to date include fimbriae, adherence and colonization factors *(ptfA, fimA, hsf-1, pfhA* and *tadD)*, iron-regulated and acquisition proteins *(tonB, hgbA, hgbB* and *fur)*, extracellular enzymes such as neuraminidase *(nanB* and *nanH)*, hyaluronidase *(pmHAS)* and superoxide dismutase *(sodA* and *sodC)*, dermonecrotoxin *(toxA)* and a variety of Outer Membrane Proteins (OMPs) such as protectins *(ompA, ompH* and *plpB)* [5-10]. These pathogenic factors help *P. multocida* to invade hosts and damage tissues.

Iron (Fe^{2+}), is an essential nutrient for both pathogenic microorganisms and their hosts [11]. It is a cofactor for a large number of important enzymes, involved in many fundamental cellular processes, including electron transfer, cell respiration, and superoxide metabolism [8]. In addition, it is a pivotal component of the innate immune response through its role in the generation of toxic oxygen and nitrogen intermediates [12]. Pathogens are involving growth, proliferation and disease processes, can not directly use Fe^{2+} from the host body, but compete with the host for Fe^{2+} by iron uptake-related proteins to fulfill their iron needs [13]. TonB protein, a periplasmic protein, plays an important role in energy-dependent transport of iron siderophores of Gram-negative bacteria as well as a virulence factor [14]. Many studies have indicated that the Gram-negative bacteria can synthesize siderophores chelate iron with high affinity in cytoplasm. This compound is chelated with Fe^{2+} of iron-binding proteins in hosts. Then the TonB protein, with the help of the ExbB/ExbD complex, transduces the energy of the cytoplasmic membrane proton-motive force (pmf), activates the siderophore receptor to allow substrate internalization into the periplasmic space and enters to cytoplasm through the specific ATP binding cassette (ABC) transporter ulterior. Consequently the pathogen is able to reunite the iron source [8,15,16]. Many other pathogenic bacteria can also directly recognize heme or heme binding proteins by outer membrane receptors for heme, and transfer the host's heme to the cytoplasm depend on the ExbB-ExbD-TonB system for energy as well as the specific ABC transporters, in this way, they obtain the iron source [17,18]. Therefore, some studies have focused on the *Haemophilus influenzae* TonB protein and have been demonstrated that TonB protein plays a crucial role in utilization of heme and produces invasive disease of *H. influenzae* [19].

In this report, we aimed to identify the capsular serotypes of *P. multocida* from duck and geese in Sichuan-Chongqing regions, China. Moreover, the analysis of genetic characteristics of TonB protein of *P. multocida* was displayed.

MATERIAL and METHODS

Samples

During the period from 15th February to 3rd December 2016, a total of 172 liver samples were collected under aseptic conditions from ducks (n = 121) with 1 to 2 months age and geese (n = 51) with 2 to 3 months age suffering from respiratory manifestations, such as nasal discharge, cough and dyspnea. The samples were collected from different farms located in Chongqing and Sichuan, China, and transferred under complete aseptic condition to the College of Science Laboratory at Rongchang Campus of Southwest University for standard bacteriological examination and molecular detection of *P. multocida*.

Bacterial Isolation and Identification

Each liver sample was plated on Luria-Bertani (LB, Oxoid, Thermo Fisher Scientific, China) agar supplemented with 5% defibrinated sheep blood. All plates were incubated at 37°C under appropriate air conditions (aerobic conditions and 90% relative humidity) for 24 h. Afterward, the isolates were purified and cultured by standard methods for rapid primary identification of *P. multocida* by PCR using the specific primers Kmt1 stated in Townsend KM et al.[20].

Capsular Types and Virulence Genes of P. multocida

The capsular types of *P. multocida* were confirmed by multiplex PCR with capsule-specific primer pairs (A and F) [21]. All isolates were analyzed with PCR in terms of the 18 virulence-associated genes which were reported specifically to *P. multocida* previously [22-24]. The primers [23] used in the virulence factors assay for *P. multocida* were synthesized commercially with finishing done by BaiLiGo Biotechnology Shanghai Co., Ltd.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *P. multocida* isolates was determined using the Kirby–Bauer disc diffusion method [25] after strains were cultured on Mueller-Hinton agar (Oxoid, Thermo Fisher Scientific, China). Amikacin (10 μg), Ampicillin (30 μg), Cefalexin (30 μg), Cefradine (30 μg), Enrofloxacin (5 μg), Gentamicin (10 μg), Kanamycin (30 μg), Amoxicillin (10 μg), Doxycycline (30 μg), Neomycin (30 μg), Erythromycin

(15 µg), Carbenicillin (100 µg), Penicillin (10 µg), Tetracycline (30 µg), Sulfisoxazole (300 µg) are the antimicrobial agents (Oxoid) which were provided from Thermo Fisher Scientific CO., LTD. As defined as the multidrug-resistance (MDR) is an acquired non-susceptibility to at least one agent in three or more antimicrobial categories [26].

Cloning of tonB Gene

DNAs of 23 *P.multacida* strains were extracted by a commercially available kit (TaKaRa Biotechnology Dalian Co. Ltd.). On the basis of complete genome sequences (NC002663.1) of *P. multocida* registered in GenBank, we designed the primer pairs (tonB-F: CCGGAATTCATGATA GATAAAAGTCGT; tonB-R: CCCAAGCTTTTAATTTGTGATT-CTGAA) to amplify the *tonB* gene, and the yielded amplified product was expected about 771bp. Each PCR contained a total volume of 50 µL, including 25 µL Premix Ex Taq polymerase (TaKaRa Biotechnology Dalian Co., Ltd), 8 µL DNA template, 2 µL of each primer (20 µmol/L) and 13 µL ddH_2O. The PCR thermal condition included 10 min initial denaturation step at 94°C, followed by 30 cycles of the following steps as 30 sec denaturation at 94°C, 45 sec annealing at 53°C, 1 min of extension at 72°C and a final 5 min extension step at 72°C. The amplicons were cloned and sequenced subsequently. PCR reaction products were ligated into the pMD-19 T vector (Takara, Dalian, China), transformed into DH5α cells, and plated onto Luria-Bertani (LB, Oxoid, Thermo Fisher Scientific, China) agar plates containing Ampicillin (100 µg/mL). For each *P. multocida* isolate, three independent colonies derived from two independent PCR reactions were sequenced to obtain a consensus sequence.

Phylogenetic Analysis of tonB Gene in P. multocida

To investigate the evolutionary relationship of *tonB* gene in *P. multocida*, phylogenetic analysis was performed by the Neighbour-Joining (NJ) and Maximum-Likelihood (ML) methods implemented in MEGA7.0 [27]. Conserved regions were determined using the Gblocks program [28]. The best-fitting nucleotide substitution model with the Akaike information criterion (AIC) score was determined using jModelTest 2.1.7 [29]. NJ and ML analyses of *P. multocida tonB* gene from different hosts were performed under the Hasegawa-Kishino-Yano + Has Invariant sites (HKY+I) model, and the robustness of the tree topology was assessed with 1,000 bootstrap replicates. NJ and ML analyses of *P. multocida tonB* gene from different sero-groups were performed under the Hasegawa-Kishino-Yano (HKY) model, and the robustness of the tree topology was assessed with 1,000 bootstrap replicates.

Phylogenetic Analysis of TonB Protein in Bacteria

We downloaded 3000 bacteria genomes from the Entrez Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), from these we sorted for 1330977 protein sequences and built a local protein database. Using cloned 23 TonB protein sequences of *P. multocida* in our study to construct a TonB Hidden Markov Model (TonB-HMM) by performing hmmbuild program of HMMER 3.0 software [30]. Retrieving the local protein database based on TonB-HMM, the screening criteria is e-value $<10^{-5}$. The screened protein sequences are considered to be predicted TonB protein sequences. All retrieved sequences were subjected to the multiple sequence alignment using L-INS-i method implemented in MAFFT7.1 software [31], and then using ML method with 500 bootstrap replicates to constructed phylogenetic tree implemented in MEGA7.0 [27].

Functional Effect of Point Mutations

To study the functional effect of point mutations on TonB protein sequence, one chosen representative TonB protein sequence was analyzed using PredictProtein (http://ppopen.rostl-ab.org/) based on the SNAP2 method, and the accuracy of results about functional effect of point mutations can be reached 82% [32].

RESULTS

Prevalence of P. multocida in Duck and Goose Clinical Samples

In this study, *P. multocida* was isolated from 23 (13.4%) out of 172 liver samples collected from duck and geese with clinical respiratory disease. Molecular identification of the recovered strains with the specific primer (Kmt1) revealed positive amplification of 457 bp fragments for all isolates *(Fig. 1)*. As the results of multiplex PCR specific for capsular antigens of *P. multocida,* 22 strains (95.7%) were found type A with a 1044 bp amplified fragment, whereas 1 strain (4.3%) was type F with 851 bp length fragment.

Antimicrobial Susceptibility

The antimicrobial susceptibility results of *P. multocida* isolates are described in *Table 1*. There was no drug which can reach to 100% efficiency in inhibition of the bacterial growth, but the isolates showed sensitivity to 50% of the antimicrobials, except sulfisoxazole and penicillin. 43.5% of the *P. multocida* isolates were found resistant to three or more drugs in different categories in this study.

Distribution of Virulence Genes

Among the 23 *P. multocida* isolates, the 18 virulence gene regions ranged in prevalence as 0% *(toxA)* to 100% *(tonB) (Table 2)*. Iron acquisition factors *(tonB*, *fur*, *hgbA* and *hgbB)*, adhesins-encoding *(ptfA* and *fimA)*, superoxide dismutases *(sodA* and *sodC)*, porin and outer membrane proteins *(plpB* and *ompH)*, neuraminidase *(nanB* and *nanH)* and hyaluronan synthase *(pmHAS)* genes were each found in more than 82.6% of the isolates. This shows that these virulence genes are highly prevalent among isolates of *P. multocida* recovered from poultry. In contrast, 5 genes *(toxA*, *tadD*, *hsf-1*, *pfhA* and *ompA)* were detected as 0% to

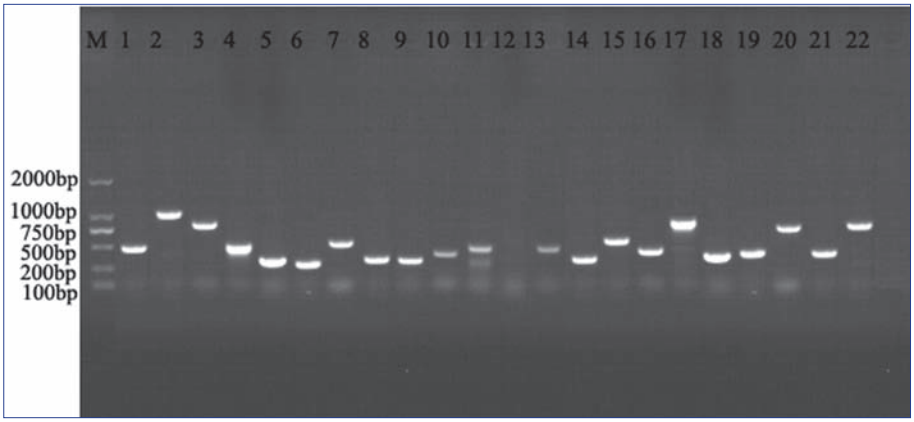

Fig 1. PCR identification, serological typing and the 18 virulence-associated genes analysis of *P. multocida* strains isolated from poultry. M: DNA Marker DL2000 (Takara, Dalian, China); 1:PCR identification for *P. multacida* (KMT gene); 2: PCR identification for capsular type A; 3: PCR identification for capsular type F; 4-21: PCR products of *ompH*, *plpB*, *ompA*, *nanB*, *nanH*, *sodC*, *sodA*, *pmHAS*, *toxA*, *ptfA*, *pfhA*, *hsf-1*, *tadD*, *fimA*, *fur*, *hgbA*, *hgbB* and *tonB* gene, respectively; 22: The complete fragment of *tonB* gene of *P. multocida*

Table 1. *The antimicrobial susceptibility patterns of P. multocida strains from poultry*

Antimicrobial	Susceptible	Non-Susceptible
Gentamicin	82.6% (19/23)	17.4% (4/23)
Kanamycin	91.3% (21/23)	8.7% (2/23)
Amoxicillin	56.5% (13/23)	43.5% (10/23)
Doxycycline	82.6% (19/23)	17.4% (4/23)
Neomycin	91.3% (21/23)	8.7% (2/23)
Erythromycin	52.2% (12/23)	47.8% (11/23)
Cefalexin	91.3% (21/23)	8.7% (2/23)
Cefradine	95.7% (22/23)	4.3% (1/23)
Sulfisoxazole	47.8% (11/23)	52.2% (12/23)
Carbenicillin	65.2% (15/23)	34.8% (8/23)
Penicillin	21.7% (5/23)	78.3% (18/23)
Amikacin	87.0% (20/23)	13.0% (3/23)
Ampicillin	56.5% (13/23)	43.5% (10/23)
Tetracycline	95.7% (22/23)	4.3% (1/23)
Enrofloxacin	91.3% (21/23)	8.7% (2/23)

Table 2. *Distribution of virulence factors of 23 strains P. multocida*

Virulence-Associated Genes	Detection (n=23)	Not Detected (n=23)
ompH	21 (91.3%)	2 (8.7%)
ompA	17 (73.9%)	6 (26.1%)
plpB	22 (95.7%)	1 (4.3%)
nanH	19 (82.6%)	4 (17.4%)
nanB	19 (82.6%)	4 (17.4%)
sodA	21 (91.3%)	2 (8.7%)
sodC	21 (91.3%)	2 (8.7%)
pmHAS	19 (82.6%)	4 (17.4%)
toxA	0 (0.0%)	23 (100%)
ptfA	19 (82.6%)	4 (17.4%)
pfhA	15 (65.2%)	8 (34.8%)
tadD	8 (34.8%)	15 (65.2%)
hsf-1	13 (56.5%)	10 (43.5%)
fimA	22 (95.7%)	1 (4.3%)
fur	22 (95.7%)	1 (4.3%)
hgbA	21 (91.3%)	2 (8.7%)
hgbB	20 (87.0%)	3 (13.0%)
tonB	23 (100%)	0 (0.0%)

73.9% percentage, and the *toxA* gene was not detected in any of the 23 studied clinical strains.

Cloning of tonB Gene

The amplified PCR product of 23 *P. multocida* isolates was 771bp as expected *(Fig. 1)*. Then the amplified product was cloned and sequenced. The 23 sequences were deposited in the GenBank database under accession numbers KY748240 - KY748250, KY623668- KY623679.

Alignment and Phylogenetic Analysis of tonB Gene in P. multocida

Phylogenetic trees with similar topologies were obtained by the two aforementioned methods (NJ and ML). As shown in *Fig. 2A*, the 22 *tonB* gene sequences of *P. multocida* deriving from different hosts fell into four distinct groups, called as Group I, Group II, Group III and Group IV. Eight isolates of this study (KY623677, KY623668, KY623670, KY623671, KY623676, KY623675, KY623669 and KY623674), poultry strain (LUCZ01000003) and *Capra aegagrus hircus* strain (AFRS01000078) belonged to Group I, whereas ovine strain (ARNZ01000046) and *Oryctolagus cuniculus* strain (NZ_MTIH01000003, NZ_MTIL01000003 and NZ_MTIF 01000003) belonged to Group II. Alpaca strain (KP660851), pig strain (CP001409), bovine strain (ARWR01000002) belonged to Group III and pig strain (CP003328) and bovine strains (ALBZ01000087, AROA01000190 and NZ_ JQEB01000010) belonged to Group IV. One rare strain from human (NBTJ01000001) did not belong to any group. As shown in *Fig. 2B*, the 22 *tonB* gene sequences of *P.multocida* deriving from different serogroups fell into two distinct groups, called as Group I and Group II. Serogroup B belonged Group II and the rest of serogroups clustered in Group I.

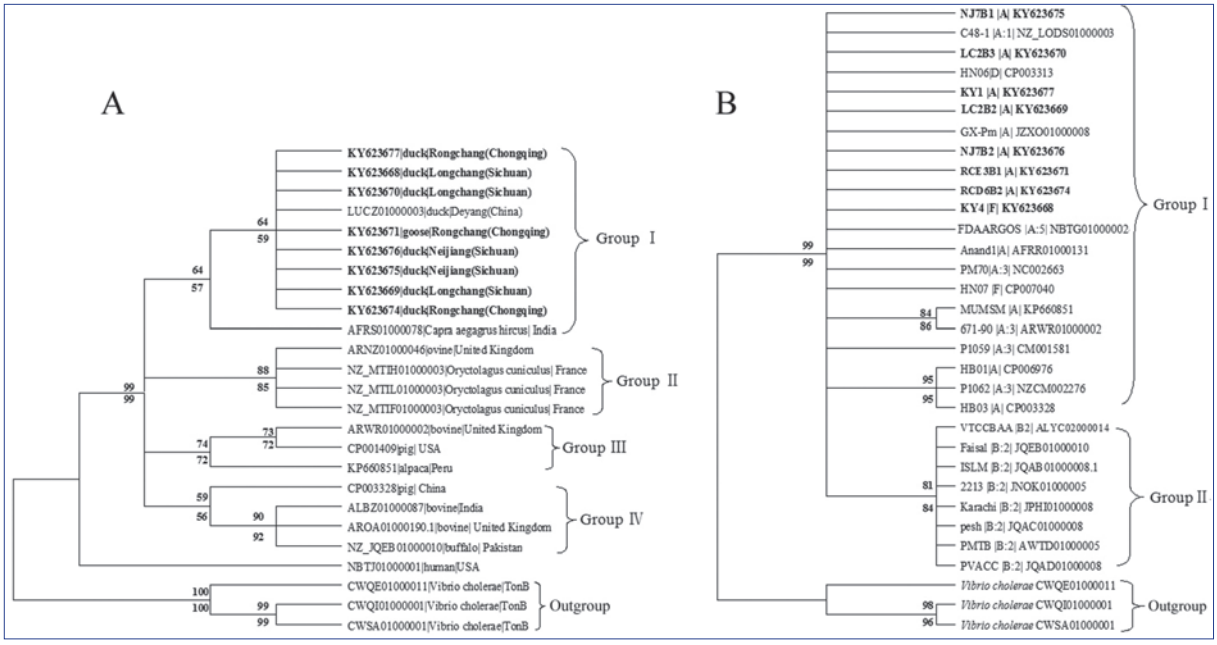

Fig 2. Evolutionary relationship of the *P. multocida tonB* gene from different hosts and different serotypes.
The NJ and ML bootstrap percentages are given above and below branches. The 8 *P. multocida* isolates from this study were indicated in bold font. The 3 *Vibrio cholerae* isolates, NC_003623, NC_004440 and NC_003839, were used as outgroups; (A) Phylogenetic tree shows genetic relationships among the *P. multocida tonB* gene from different hosts; (B) Phylogenetic tree shows genetic relationships among the *P. multocida tonB* gene from different serotypes

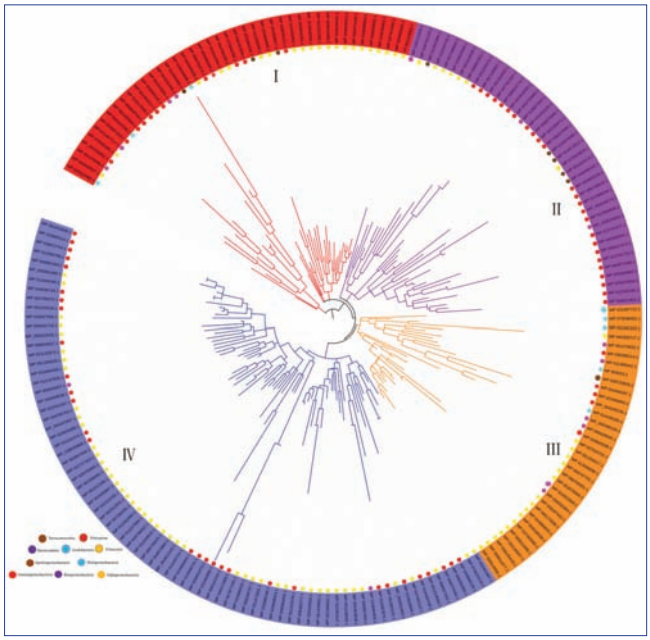

Fig 3. Phylogenetic tree of 196 typically bacterial TonB protein sequences. The numbers represent GenBank accession numbers. Different colors in the tree represent different groups of TonB: Red represents Group I, purple represents Group II, orange represents Group III, blue represents Group IV. The trees are based on the MAFFT-derived multiple sequence alignments (MSA). The tree was drawn with the iTOL program

Phylogenetic Analysis of TonB Protein in Bacteria

We obtained 302 significant sequences (E-value $<10^{-5}$) after retrieving the local protein database based on the TonB-HMM model, after that, 196 sequences were TonB protein sequences through screening and correcting. According to the phylogenetic tree *(Fig. 3)*, 196 sequences derived from 6 phyla, which contains *Proteobacteria* (187 sequences) and 9 sequences distributed among *Firmicutes, Nitrospirae, Acidobacteria, Bacteroidetes* and *Verrucomicrobia*. In *Proteobacteria*, 88 sequences were derived from *Alphaproteobacteria*, 71 sequences from *Gammaproteobacteria*, 13 sequences from *Betaproteobacteria*, 8 sequences from *Epsilonproteobacteria* and 7 sequences from *Deltaproteobacteria*. The proportions of them were 47.06%, 37.97%, 6.95%, 4.28% and 3.74%, respectively. So we knew that the bacteria shared a high similar sequence with the *P. multocida* TonB protein sequence were mainly concentrated in *Alphaproteobacteria* class of *Proteobacteria* phylum.

Multiple Sequence Alignment and Point Mutation Analysis

The phylogenetic tree was constructed with 4 lineages, as shown in *Fig. 4A*. According to the *Fig. 4A*, the -xSSGx- and -YP- motifs were the best conserved portion in these 4 lineages although there was a great variation of TonB protein sequences among different species. The -LD- motif existed in cluster 1, 2 and 4, while the -xA[A/V]Lx- motif distributed in cluster 1, 2 and 3, and these motifs were relatively conserved. The protein sequence of representative cloned strain (KY748245) was analyzed for the point mutation. The results showed that 3 amino acids of -xSSGx- motif in this cloned strain were highly conserved, and it is known if they are replaced by any

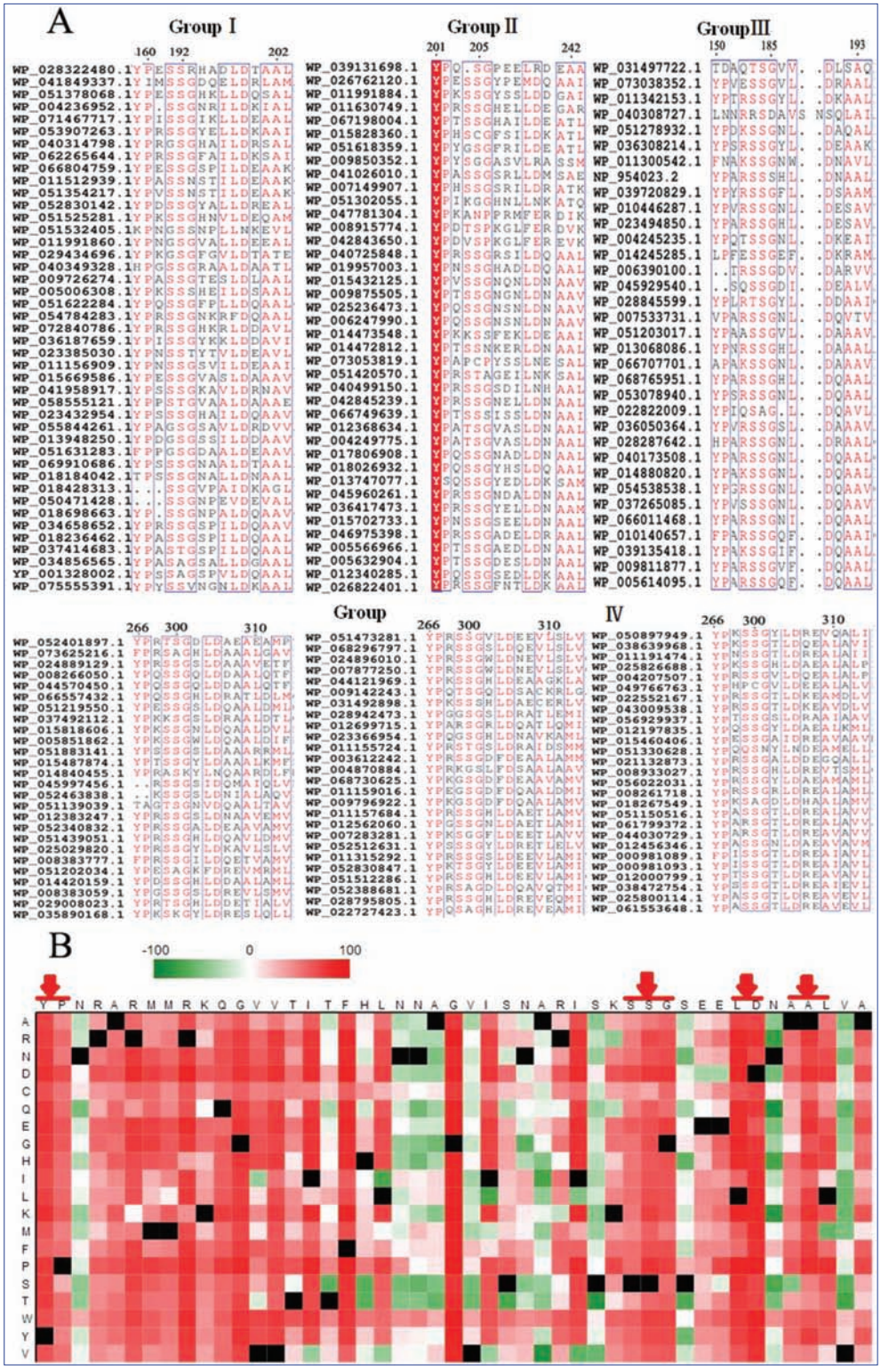

Fig 4. Alignment of amino acid sequences of bacterial TonB in each group and analysis of the effect of point mutations of KY748245. A: The -xSSGx- and -YP- motifs existed in four groups, the -LD-motif existed in Group I, II, IV, and the -xA[A/V]Lx-motif existed in Group I, II , III; B): Deep red (>50): Have great influence on function if replaced; Green and White (<50): Have relatively small influence on function if replaced; Black: Wild type

amino acid, there must be some serious impacts on the function of TonB protein in bacteria *(Fig. 4B)*. In addition, the amino acids in -LD- and -xA[A/V]Lx- motifs were also highly conserved *(Fig. 4B)*.

DISCUSSION

As an infectious pathogen, *P. multocida* has great harm to a wide range of hosts and can cause many diseases. Parallel with the developments on the research of *P. multocida*, it has been almost completely controlled. Nevertheless, the pathogenic mechanism is currently not well understood, and thus it still restricts the development of livestock and poultry breeding and causes severe economic losses [9,33]. In this study, 23 strains of *P. multocida* isolated from poultry in Chongqing and Sichuan, China were tested for capsular antigens and antimicrobial susceptibility. The results showed that the capsular types of isolates were predominantly type A which is similar to that it was usual reported for *P. multocida* in China and other countries [34,35]. But we found one isolate of *P. multocida* was type F which is similar to the findings that reported previously [33]. Meanwhile, in these two provinces, antibiotic therapy is still an effective tool in the treatment of infections caused by *P. multocida*. Similarly, the potential threat of such multi resistant bacteria in food producing animals should not be neglected.

The *toxA* gene encodes dermonecrotic toxin, but the *toxA* gene is not detected in this study. This result may indicate that the avian strain or serotype A of *P. multocida* is not easy to carry the *toxA* gene. Some research suggested that the *toxA* gene is more significantly associated with serotype D, compared to serotype A [33,36]. We investigated iron-regulated and

iron-acquisition related genes *(tonB*, *fur*, *hgbB*, *hgbA)* irrespective of the animal clinical status and found that all isolates had the *tonB* gene similar to the findings reported previously [37]. In a past study [38], the *tonB* gene encoded an energy transfer protein.

TonB protein is a periplasmic protein prevalent in a large number of Gram-negative bacteria, which is believed to be responsible for the import of ferric iron complexes across the outer membrane [39,40]. Not only TonB protein activates outer membrane to make substrates into the periplasm as an energy transduction component [41], but also plays a role as an important virulence factor to help Gram-negative bacteria to obtain nutrients. In this study, we attempted to amplify and clone the *tonB* gene from 23 isolated strains of *P. multocida*, and then to explore the evolutionary relationship of the *P. multocida tonB* genes with different host and serotypes have. It was showed in *Fig. 2*, *P. multocida tonB* gene is present in different hosts and also in different serotypes. The phylogenetic tree displayed that these *tonB* genes have close genetic relationship and there is a high similarity (92.9%-100%) among these *tonB* genes. A similar distribution of certain genes, regardless of the host species of *P. multocida* serotype, may suggest that the selection of factors that present cross-protection as candidates for vaccine development [33,42]. TonB protein is generally conserved among species and has a high immunogenicity. Therefore, TonB protein potentially serves as vaccine candidates.

To study the evolutionary relationship of TonB protein in bacteria, a TonB Hidden Markov Model named TonB-HMM was built. HMMER2.3.2 software was used to retrieve the local protein database based on this model to analyze the genetic characteristics of TonB protein in *P. multocida* and other bacteria. Based on the phylogenetic analysis *(Fig. 3)*, we found that TonB protein of *P. multocida* shared a high homology with TonB protein of *Alphaproteobacteria* and *Gammaproteobacteria* classes in *Proteobacteria* phylum. Multiple sequences alignment was performed and the results reported in *Fig. 4A* show that the -xSSGx- and -YP- motifs were the best conserved portion in these four groups, which is consistent with the data reported previously about the -xSSGx- motif as a highly conserved motif in TonB protein [40]. It is well known that there is a certain functional value may exist in conserved sequences and it was reported that the -xSSGx- motif might play a role in TonB protein and receptor recognition [39]. Previous studies on -YP- motif had emphasized that C-terminal domain of TonB protein was closed to the cobalamin transporter BtuB [43]. In addition, the -YP- motif contacted with ferricytochrome receptor (FhuA) and the TonB-box [44]. Moreover, we found that the -LD- and -xA[A/V]Lx- motifs were also relatively conserved, meanwhile, the cloned strain KY748245 was predicted for point mutation. The results suggest that the amino acid of the -LD- and -xA[A/V]Lx- motifs have strong conservative, therefore, to some extent, the function of TonB protein can be further explored by studying the point mutation of the -LD- and -xA[A/V]Lx- motifs, which is highly significant. Thus, we can attempt to perform point mutation, substitution and destruction of these conserved amino acid and motifs to change the function of TonB protein. However, as conserved amino acid and motifs are changed, we are not sure whether it can make pathogenicity weaken, so further studies are required.

In conclusion, we successfully isolated a total of 23 strains of *P. multocida* from poultry, of which 22 strains were identified as serogroup A and one strain was serogroup F. The research provided information regarding the distribution of virulence genes of *P. multocida* strains isolated from poultry in Chongqing-Sichuan regions, China. The *P. multocida tonB* gene was found a high frequency in this study and it was not specific to a host or a serogroup. The genetic evolutionary relationship with TonB protein of Gram-negative bacteria was elucidated by phylogenetic analysis, and conserved motifs of TonB protein were predicted. We can try to study the performance of these motifs from various perspectives, which may be further extended for functional studies of TonB protein in bacteria and provides a reference for the researches of drug target in *P. multocida*.

Acknowledgments

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Research Article

Effect of Guanidine Acetic Acid Addition to Corn-Soybean Meal Based Diets on Productive Performance, Blood Biochemical Parameters and Reproductive Hormones of Laying Hens

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Abstract

The present study was conducted to investigate the effects of GAA addition to corn-soybean meal based diets on productive performance, blood biochemical parameters and reproductive hormones of laying hens. Two hundred laying hens were used in a completely randomized design with 5 treatments and 4 replicates (n=10 birds). Birds received basal diets containing 0 (negative control), 0.057, 0.114 and 0.171% GAA/kg of diet. A diet containing 2% meat meal was also considered as positive control. The data for productive performance was recorded during 84 d and blood samples were collected to evaluate the blood biochemical parameters including triglycerides, high and low density lipoproteins, aspartate aminotransferase and alanine aminotransferase and nitric oxide and reproductive hormones (LH and FSH) at 42 and 84 d. Results showed that GAA addition to diet had not significant effects on productive performance but 0.171% GAA addition reduced egg weight (P<0.05) compared with control groups. Also, 0.114% GAA addition to diet increased levels of LH and FSH compared with control groups at 42 and 84 days. It can be concluded that GAA addition to diet is not appropriate strategy for improving performance of laying hens.

Keywords: *Creatine, Guanidine acetic acid, Laying hens, Nitric Oxide, Productive performance, Reproductive hormones*

Mısır-Soya Küspesi Diyetine Eklenen Guanidino Asetik Asitin Yumurta Tavuklarının Üretim Performansı, Kan Biyokimyasal Parametreleri ve Üreme Hormonlarına Etkisi

Özet

Bu çalışma GAA'nın mısır soya fasulyesi ağırlıklı rasyonlara eklenmesinin üretim performansı, kan biyokimyasal parametreleri ve yumurta tavuklarının üreme hormonları üzerindeki etkilerini araştırmak amacıyla yürütülmüştür. Çalışma tamamen rastgele edilmiş bir tasarım ile 5 grup ve 4 alt grup (n = 10 tavuk) iki yüz yumurta tavuğu ile yürütülmüştür. Tavuklar 0 (negatif kontrol), 0.057, 0.114 ve %0.171 GAA/kg içeren bazal rasyon ile beslenmiştir. Ayrıca rasyonda %2 et unu içeren bir de pozitif kontrol grubu oluşturulmuştur. Üretim performans verileri 84 gün boyunca kaydedildi ve trigliseridler, yüksek ve düşük yoğunluklu lipoproteinler, aspartat aminotransferaz, alanin aminotransferaz, nitrik oksit ve üreme hormonları (LH ve FSH) başta olmak üzere kan biyokimyasal parametrelerini değerlendirmek için 42 ve 84. günlerde kan numuneleri alındı. Sonuç olarak GAA'nın tavuk rasyonuna ilave edilmesiyle, üretim performansı üzerinde anlamlı bir etkisinin olmadığı, %0.171 GAA katkısının kontrol grubuna kıyasla yumurta ağırlığını azalttığı (P<0.05) görüldü. Ayrıca, rasyonda %0.114 GAA ilavesi ile kontrol gruplarına kıyasla LH ve FSH düzeylerinde artış görülmüştür. GAA'nın rasyona eklenmesinin yumurta tavuklarında performansını arttırmak için uygun bir strateji olmadığı sonucuna varıldı.

Anahtar sözcükler: *Kreatin, Guanidin asetik asit, Yumurta tavuğu, Nitrik Oksit, Üretim performansı, Üreme hormonları*

INTRODUCTION

Guanidine acetic acid (GAA) is synthesized from arginine and glycine and it is precursor for creatine synthesis [1]. Studies have shown that by 50% of daily creatine requirements is supplied through *de-novo* synthesis and the rest part by the feed [2]. It has been known lack of creatine in corn-soybean meal based diets [3,4]. It seems

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that lack of creatine reduces cell performance and finally productive performance in animals because of its role in metabolism.

Studies have shown that GAA addition to diets increases its conversion to creatine and this conversion was evidenced by increase in muscle and blood creatine [5-7]. GAA addition to diet is beneficial because it has much amounts arginine which may be spared [8,9]. Studies have shown that GAA spares arginine in deficient-arginine diets and it additionally improves growth performance in enough-arginine diets [7]. Earlier studies have shown the importance of arginine for nitric oxide and protein synthesis, growth and development in vertebrata [10,11]. Nitric oxide releases gonadotropin-releasing hormone (GnRH) by activating pituitary nitric oxide synthase which finally influences FSH and LH hormones [12]. Studies have indicated that FSH is involved in the recruitment of new follicles into the ovarian hierarchy and also the maturation and rapid growth of these new follicles [13,14]. A study has shown that FSH and LH hormones increase oviduct secretions and egg weight and also improve feed conversion ratio in laying hens [15]. It was reported a positive correlation between LH and ovulation [16]. Considering sparing effect of arginine by GAA, Basiouni [17] stated that arginine increases LH hormone secretion and subsequently improves productive performance in laying hens. On the other hand, arginine reduces abdominal lipid and balances lipid storage by nitric oxide synthesis [18]. Yang *et al.*[19] showed that dietary inclusion of arginine significantly reduced levels of liver aspartate amino-transferase in laying hens, implicating that arginine reduces liver damages. GAA is able to sparing body pool of arginine which is used for creatine synthesis [20] since it is converted to creatine in bird's kidney and liver [21]. Any study has been not still investigated effects of GAA on productive performance and reproductive hormones in laying hens. This study was conducted to investigate of several hypotheses including 1) GAA, as creatine precursor, acts and may improve energy requirements, if needs are faulted. 2) GAA is consisted of arginine and glycine and considering previous studies; does it spares and produces arginine in laying hens? 3) Considering relation between GAA and nitric oxide and as well as effects of nitric oxide on reproductive hormones and lipids, does it improves lipid and reproductive hormones by nitric oxide? Thus, the present study was conducted to investigate the effects of GAA addition to corn-soybean meal based diets (lack of creatine) on productive performance, blood biochemical parameters and reproductive hormones of laying hens.

MATERIAL and METHODS

Animal and Feed Material

All the used experimental protocols were approved by the guidelines of the Animal Ethics Committee of Science and Research University (Tehran, Iran). This experiment was conducted in house hen poultry farm placing in Shahryar town (Tehran-Iran). Two-hundred Hy-Line W-36 laying hens, 29 weeks of age and with mean body weight of 1410±100 g, were allocated to a completely randomized design with 5 treatments and 4 replicates (n=10 birds). One week adaption period was considered and birds were 30-old-week of age in start of experiment. The experimental period was lasted for 12 weeks. The rearing conditions were similar for all birds. Laying hens were kept in a layer house with 2 hens in each battery cage (50×50 cm) and each 5 cage were considered as a replicate. The birds were maintained under 16L:8D lighting program at all the experiment. Housing temperature and relative humidity were kept at 18°C and 45-50%, respectively. Water and feed were *ad libitum* supplied at all the study by drinkers and feeders, respectively. CreAmino®, produced by Evonic Industry Co, was used as GAA source which was containing 96% GAA, 1% water and 1% starch. Nutritional matrix of CreAmino® was as follows; 100% digestibility, 221% crude protein, 77% arginine and 83.000 kcal/kg AMEn. Birds received basal diets supplemented with different levels of GAA (0, 0.057, 0.114 and 0.171%). Two control diets, negative and positive, were also considered. Experimental treatments were as follows; 1) corn-soybean meal based diets without supplement as negative control, 2) corn-soybean meal based diets containing 0.057% GAA, 3) corn-soybean meal based diets containing 0.114% GAA, 4) corn-soybean meal based diets containing 0.171% GAA and 5) diets containing 2% meat meal as positive control. Experimental diets were prepared on the basis Hy-Line W-36 catalogue. The ingredients and composition of the basal diet are presented in *Table 1*. The proximate analyses of some nutrients were performed according to Association of official Analytical Chemists, or AOAC [22]. The amounts calculated of GAA were firstly mixed small amounts of the basal diet as a small batch, and then added to the calculated amount of the basal diet to obtaining a homogenous diet. The feed was prepared in mash form for all birds at same place.

Productive Performance

Egg production (EP), feed intake (FI), and egg weight (EW) was daily recorded during 1-84 days of trial, from each cage and egg mass (EM: EP×EW/100) feed conversion ratio (FCR: FI/EM) and were biweekly calculated. Body weight (BW) was measured at start and end of trial to measure the changes.

Blood Sampling and Measurement of Hormones

At 42 and 84 days of trial, blood samples (3 mL) were collected from 1 bird each replicate and centrifuged at 2500×g for 15 min. Levels of LH (CAT No. ZB-0014-Ch9648), FSH (CAT No. ZB-0012-Ch9648), nitric oxide (CAT No. ZB-NO-48A, V406), aspartate aminotransferase (CAT No. ZB-0143-Ch9648) and alanine aminotransferase (CAT No. ZB-0131-Ch9648) were measured using ZellBio® GmbH

Table 1. *Composition of experimental diets*

Ingredient (%)	Negative Control	0.057% GAA	0.114% GAA	0.171% GAA	Positive Control
Corn	63.60	63.54	63.48	63.42	64.00
Soybean meal	23.00	23.00	23.00	23.00	21.00
GAA	0.00	0.057	0.114	0.171	0.00
Meat meal	0.00	0.00	0.00	0.00	2.00
Soybean oil	1.20	1.203	1.206	1.209	0.80
Dicalcium phosphate	1.20	1.20	1.20	1.20	1.20
Limestone	9.60	9.60	9.60	9.60	9.60
Salt	0.20	0.20	0.20	0.20	0.20
Soda	0.25	0.25	0.25	0.25	0.25
Potassium carbonate	0.25	0.25	0.25	0.25	0.25
Premix[1]	0.50	0.50	0.50	0.50	0.50
Methionine	0.16	0.16	0.16	0.16	0.16
Lysine	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.02	0.02	0.02	0.02	0.02
***Nutrient analysis* (calculated)**					
ME, kcal/kg	2571.39	2571.39	2571.39	2571.39	2571.39
Crude protein, %	15.93	15.93	15.93	15.93	15.93
Crude fiber, %	15.93	15.93	15.93	15.93	15.93
NFE, %	3.90	3.90	3.90	3.90	3.90
Anion-cation, kg/mEq	202.15	202.15	202.15	202.15	202.15
Ca, %	3.89	3.89	3.89	3.89	3.89
Available P, %	0.29	0.29	0.29	0.29	0.29
Sodium, %	0.16	0.16	0.16	0.16	0.16
Methionine,%	0.39	0.39	0.39	0.39	0.39
Lysine, %	0.74	0.74	0.74	0.74	0.74
Methionine+ cysteine,%	0.62	0.62	0.62	0.62	0.62
Threonine %	0.53	0.53	0.53	0.53	0.53

[1] Provided per kilogram of diet: Vit. A, 5000 IU; Cholecalciferol, 750 IU; Vit. E, 7.5 mg; Menadione, 0.63 mg; Thiamine, 0.25 mg; Riboflavin, 1.60 mg; Pyridoxine, 0.500 mg; Vit. B_{12}, 4.0 µg; Niacin, 12.5 mg; Calcium pantothenate, 1.8 mg; Butylated hydroxytoluene, 63 mg, Iron, 44 mg; Iodine, 1.2 mg; Cobalt, 0.36 mg; Selenium, 0.24 mg

commercial kits and by ELISA method as recommended by Producer Company. The serum concentrations of triglycerides, high and low density lipoprotein were measured by Pars Azmun commercial kits (Tehran-Iran).

Statistical Analysis

A completely randomized experimental design (CRD) were applied and the data were subjected to statistical analysis using analysis of variance (ANOVA) appropriate for a. When significant effects were detected by ANOVA, treatment means were compared using Duncan's multiple range test. All statistical analyses were performed with SAS [23]. Differences were considered significant at $P<0.05$. All of the parameters were analyzed as follows;

$$Yij = \mu + Ti + eij$$

Where Yij is the individual observation, µ is the overall mean, Ti is the effect of treatment, and eij represents the random error.

RESULTS

Tables 2, 3, 4, 5 and *6* show the effects of GAA addition to diet on productive performance. During experiment and in total**,** the added different levels of GAA to diet did not change FI, FCR, EP and EM in comparison with positive and negative controls ($P>0.05$); showing that dietary inclusion of meat meal and GAA have not significant effects on FI, FCR, EP and EM of laying hens. EW was reduced from 2th week to 12th week in laying hens fed the 0.171% GAA compared with other groups ($P<0.05$). Dietary inclusion of 0.114% GAA to diet significantly ($P<0.05$) increased levels of LH and FSH compared with negative and positive controls at 42 and 84 d *(Table 7)*. Blood biochemical parameters were not influenced by GAA addition supplement ($P>0.05$; *Table 8)*.

DISCUSSION

Findings showed that GAA addition to diet had not significant effects on productive performance but 0.171% GAA addition decreased egg weight from 2th week to

Table 2. *Effects of GAA addition to diet on FI (g) of laying hens at different periods*

Groups	Negative Control	Positive Control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
1-14d	87.75±4.65	86.49±5.61	86.25±6.01	86.31±5.73	81.75±3.53	0.844	0.321
15-28d	89.38±3.77	86.75±5.70	86.75±3.37	87.50±2.87	85.50±4.50	0.457	0.656
29-42d	86.38±4.30	85.38±6.20	81.50±5.42	86.63±3.24	82.38±4.37	0.129	0.737
43-56d	87.00±2.77	84.63±5.52	83.00±4.20	85.75±2.52	87.25±1.98	0.131	0.707
57-70d	91.21±2.02[a]	89.09±3.12[a]	84.50±4.50[b]	88.00±3.42[a]	88.50±2.13[a]	0.003	0.589
71-84d	93.88±5.79	92.34±4.51	88.19±4.55	92.63±3.10	93.23±3.86	0.093	0.719
1-84d	89.26±2.55	87.40±4.37	85.03±3.71	87.80±1.26	86.43±1.61	0.347	0.675

***SEM:** standard error of means. Superscripts (a, b) shows significant differences at each row ($P<0.05$). The data are presented as mean ± standard deviation*

Table 3. *Effects of GAA addition to diet on EP (%) of laying hens at different periods*

Groups	Negative Control	Positive Control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
1-14d	88.04±7.93	82.11±7.77	89.64±3.70	88.21±2.82	87.14±2.95	0.091	0.926
15-28d	86.07±9.12	83.69±5.25	87.23±4.31	86.07±4.41	85.71±4.48	0.656	0.900
29-42d	86.43±8.11	84.94±7.28	86.19±4.68	86.13±6.39	85.60±5.10	0.979	1.12
43-56d	82.32±7.12	82.38±5.03	81.43±2.16	84.46±5.31	87.50±4.56	0.132	0.841
57-70d	84.11±10.23	84.40±4.89	84.59±2.23	83.04±8.16	85.71±4.18	0.952	0.998
71-84d	74.82±12.17	75.45±7.07	74.90±10.18	73.75±9.89	80.18±9.94	0.731	1.53
1-84d	83.63±7.93	82.11±2.35	83.95±2.58	83.61±5.50	85.30±3.63	0.942	0.991

SEM: *standard error of means. The data are presented as mean ± standard deviation*

Table 4. *Effects of GAA addition to diet on EW (g) of laying hens at different periods*

Groups	Negative Control	Positive Control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
1-14d	55.60±0.75^{a}	55.61±1.09^{a}	55.87±0.89^{a}	55.14±0.32^{a}	53.94±0.80^{b}	0.0003	0.164
15-28d	55.80±0.96^{a}	55.53±0.77^{a}	56.12±0.72^{a}	56.34±0.62^{a}	54.87±1.18^{b}	0.019	0.154
29-42d	56.44±1.02^{a}	56.22±1.09^{a}	56.47±0.59^{a}	56.97±0.84^{a}	54.82±1.00^{b}	0.0006	0.178
43-56d	56.39±1.42^{a}	56.30±1.12^{a}	56.85±0.59^{a}	56.97±0.76^{a}	55.04±0.71^{b}	0.003	0.141
57-70d	57.27±1.03^{a}	57.57±0.99^{a}	57.82±0.71^{a}	58.21±0.98^{a}	55.16±0.72^{b}	0.0009	1.74
71-84d	58.03±1.11^{a}	58.03±1.18^{a}	57.96±1.36^{a}	58.93±0.975^{a}	56.80±0.836^{b}	0.0124	1.53
1-84d	56.58±0.90^{a}	56.54±0.99^{a}	56.82±0.57^{a}	57.09±0.56^{a}	55.26±0.670^{b}	0.036	0.209

SEM: *standard error of means. Superscripts (a, b) shows significant differences at each row (P<0.05). The data are presented as mean ± standard deviation*

Table 5. *Effects of GAA addition to diet on EM (g/hen/d) of laying hens at different periods*

Groups	Negative Control	Positive Control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
1-14d	48.96±4.69	45.66±2.56	50.08±3.34	48.63±2.67	47.00±3.35	0.469	0.537
15-28d	48.02±5.22	46.47±3.34	48.95±2.75	48.23±2.40	47.02±2.51	0.814	0.400
29-42d	48.78±4.68	47.75±3.10	48.67±4.21	49.06±2.93	46.92±4.85	0.441	0.619
43-56d	46.42±4.04	46.21±2.55	46.29±3.25	48.11±1.45	48.16±2.46	0.446	0.455
57-70d	48.16±5.61	48.58±2.37	48.90±2.53	48.33±1.64	48.13±4.17	0.991	0.536
71-84d	43.41±6.75	43.78±5.31	43.33±3.46	43.46±5.85	45.54±3.14	0.908	0.826
1-84d	47.30±4.40	46.40±1.90	47.70±1.18	47.62±1.84	47.13±2.74	0.982	0.535

SEM: *standard error of means. The data are presented as mean ± standard deviation*

Table 6. *Effects of GAA addition to diet on FCR of laying hens at different periods*

Groups	Negative Control	Positive Control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
1-14d	1.79±0.17	1.89±0.25	1.72±0.15	1.77±0.10	1.73±0.10	0.304	0.026
15-28d	1.86±0.20	1.86±0.13	1.77±0.11	1.81±0.15	1.81±0.17	0.398	0.028
29-42d	1.77±0.12	1.85±0.10	1.77±0.10	1.76±0.12	1.75±0.13	0.121	0.038
43-56d	1.87±0.15	1.83±0.12	1.79±0.11	1.78±0.07	1.81±0.09	0.280	0.018
57-70d	1.89±0.23	1.83±0.11	1.72±0.11	1.82±0.14	1.83±0.10	0.656	0.023
71-84d	2.16±0.32	2.10±0.22	2.03±0.31	2.13±0.28	2.04±0.28	0.883	0.044
1-84d	1.88±0.16	1.88±0.07	1.78±0.10	1.84±0.10	1.83±0.10	0.911	0.020

SEM: *standard error of means. The data are presented as mean ± standard deviation*

Table 7. *Effects of GAA addition to diet on reproductive hormones (mIU/mL) and nitric oxide (NO) (µmol/mL) of laying hens at 42 and 84 d*

Groups	Negative control	Positive control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
42 d							
LH	5.37±0.24^{b}	5.31±0.33^{b}	5.44±0.29^{b}	6.55±0.83^{a}	5.71±0.45^{ab}	0.014	0.142
FSH	4.93±0.34^{b}	4.84±0.58^{b}	5.33±0.25^{ab}	5.92±0.70^{a}	4.84±0.58^{b}	0.041	0.139
NO	216.80±24.72	216.00±14.17	225.00±19.97	272.80±58.09	232.80±17.15	0.113	7.84
84 d							
LH	7.12±0.37^{b}	7.52±0.88^{b}	7.97±0.42^{ab}	8.76±0.43^{a}	7.96±0.47^{ab}	0.009	0.166
FSH	6.61±0.43^{c}	7.06±0.33^{b}	7.36±0.21^{b}	8.16±0.79^{a}	7.10±0.58^{b}	0.009	0.155
NO	142.30±56.96	155.80±53.66	153.50±63.61	207.30±33.15	168.10±35.50	0.353	12.49

SEM: *standard error of means. Superscripts (a-c) shows significant differences at each row (P<0.05). The data are presented as mean ± standard deviation. Each IU LH and FSH is 0.13369 and 0.1138 mg, respectively*

Table 8. *Effects of GAA addition to diet on lipid profile (mg/dL) and enzymes (ng/mL) of laying hens at 42 and 84 d*

Groups	Negative control	Positive control	0.057% GAA	0.114% GAA	0.171% GAA	P	SEM
42 d							
Triglycerides	279.4±23.39	235.10±16.38	224.70±24.00	255.10±19.00	257.30±40.80	0.0749	6.78
HDL-C	40.15±3.73	41.65±2.92	35.83±3.52	41.48±4.68	39.63±6.45	0.381	1.00
LDL-C	84.48±24.72	82.48±14.17	76.60±12.17	84.58±18.09	76.35±17.15	0.685	6.82
AST	175.80±67.31	158.00±66.99	111.50±39.91	147.80±7.50	135.30±34.73	0.445	10.88
ALT	48.25±2.63	51.00±8.90	44.75±3.20	51.00±6.63	53.50±4.93	0.298	1.33
84 d							
Triglycerides	246.70±18.14	280.40±36.47	233.35±57.69	227.00±42.84	242.40±34.66	0.404	9.02
HDL-C	42.70±6.01	42.23±2.93	40.38±6.50	38.60±4.96	37.20±3.53	0.492	1.09
LDL-C	88.25±10.10	94.33±8.76	86.77±17.33	77.25±12.33	79.40±9.40	0.303	3.77
AST	69.75±16.74	64.25±15.84	58.00±15.12	51.75±28.76	56.75±11.59	0.582	4.41
ALT	46.50±5.00	46.75±0.957	46.75±4.99	45.25±3.77	47.75±3.59	0.931	0.805

SEM: *standard error of means. Superscripts (a-c) shows significant differences at each row (P<0.05). The data are presented as mean ± standard deviation.* ***AST:*** *aspartate aminotransferase,* ***ALT:*** *alanine aminotransferase*

12th week in laying hens (P<0.05) compared with other groups. Our results are in agreement with the reports of other studies that showed GAA addition to diets had not significant effects on FI of broiler chicks [24,25]. However, Michiels *et al.*[7] observed only slightly increase in FI of broiler chicks fed with GAA. It seems that GAA addition has not significant effect on FI in all birds. FCR was no influenced by GAA treatments and positive control (2% meat meal); showing that creatine supplementing from each source had not significant effect on the mentioned parameter. Tossenberger *et al.*[24] stated that GAA addition to diet had not significant effect on FCR of broiler chicks. Other studies have shown that GAA addition to diet lowered FCR in broiler chicks [2,5,25-27]. Metwally *et al.*[28] showed that GAA addition to diet could spare 50 kcal metabolizable energy and 0.5% crude protein. Ringle *et al.*[25] showed that GAA addition to corn-soybean meal diets may have beneficial effects in improving growth and FCR. Studies have shown beneficial effects of GAA on FCR, but all the studies are conducted in broiler chicks. So far, any study is not conducted to investigate the effects of GAA on productive performance of laying hens. However, exact mechanism of the effects of GAA in broiler chicks is not still known. It is shown that GAA addition to diet has beneficial effects in improving the growth, muscle mass and creatine synthesis [7]. This study was conducted in laying hens and GAA had not significant effect on body changes. Weight changes were by 30 g in all groups in the present study. It seems that GAA induces its effects on FCR by increasing muscle mass and subsequently improves FCR. Increase in body weight in laying hens is not desirable and on the basis our results, GAA has not any role in improving EP, thus GAA cannot increase FCR in laying hens.

The second status was that GAA can spare arginine and help to protein synthesis which finally increases EP and EM. Bryant-Angeloni [29] showed that GAA synthesizes protein by sparing arginine. Considering sparing effect of arginine by GAA, Basiouni *et al.*[30] showed that dietary inclusion of 1.5% digestible arginine to laying hen diet

increased production from 52% to 67.86%. Silva *et al.*[31] reported that dietary inclusion of arginine increased EP in broiler breeders. Sparing effect of arginine by GAA may be reason for improving performance in birds which it was found in studies on broiler chicks [29]. In the present study, it seems that GAA could not spare arginine and subsequently did not influence EP. Other status was that GAA addition to diet increases EP by increasing NO which subsequently increases reproductive hormones [12] and the hormones increase EP [15]. In the current study NO was not affected by dietary treatments and EP was not increased. However, an increase in FSH and LH was seen in 0.114 GAA treatment which NO is not responsible for it, because NO levels is constant.

EW was significantly reduced in laying hens fed the 0.171% GAA compared with other groups. In other words, the lowest levels of GAA had not significant effects on EW, while the highest levels reduced it. EW is a parameter which affects by diets protein and amino acid level [32] and the arginine role is accepted as increaser the protein synthesis [33]. The mechanism of action is not known.

Dietary inclusion of 0.114% GAA increased LH and FSH compared with controls at 42 and 84 d. Investigation of relation between nitric oxide and GAA was one of purposes of the present study. It is well known that nitric oxide releases gonadotropin-releasing hormone (GnRH) by activating pituitary nitric oxide synthase which finally influences FSH and LH hormones [12]. It can be found from *Table 7* that nitric oxide was numerically higher in 0.114% GAA group, but the differences were not significant. It seems that nitric oxide cannot be reason for increase in reproductive hormones. Sharifi *et al.*[34] showed that arginine addition to broiler chicks diets significantly increased nitric oxide level. GAA is synthesized from arginine and glycine, it was expected that GAA addition to diet increase nitric oxide level, but such result was not found. It may be attributed to the GAA bioavailability, bird type and age.

Results indicated that GAA addition to diet had not significant effect on blood lipid profiles and liver enzymes. Studies have not still investigated effects of GAA on blood lipid profiles and liver enzymes. Considering studies on arginine, Al-Daraji *et al.*[35] showed that dietary inclusion of arginine reduced the serum concentration of triglyceride in broiler chicks. Blood parameters are important, especially in meat-type birds [36]. Studies have been shown that increase in VLDL is criteria for showing the increased body lipid [37]. Other study reported that the serum concentration of triglyceride is criteria for showing body lipid [36]. In the present study, lipid levels were not influenced by GAA. Nitric oxide is known to have roles in lipid metabolism and thus arginine reduces abdominal lipid and indirectly balances lipid storage by nitric oxide synthesis [18]. In the present study, since nitric oxide level was not affected by GAA supplementing, thus it is reasonable that lipid profile is not also affected. Considering liver enzymes in serum, Yang *et al.*[19] showed that dietary inclusion of arginine significantly reduced levels of liver aspartate aminotransferase in laying hens. The increased aspartate aminotransferase in blood is considered as index for muscle and liver damages, which its levels are different in various birds [38]. The increased alanine amino transferase level is considered as index for tissue damages [38]. Thus, the increased blood levels of these enzymes implicates on tissue and liver damages. In the present investigation, the both levels were not influenced by GAA addition; showing that GAA has not positive or negative effects on liver enzymes.

In conclusion, the present study was conducted to investigate the relation among GAA addition with nitric oxide, reproductive hormones and lipid profile in laying hens. Findings showed that GAA could not improve performance, nitric oxide level and blood biochemical parameters. Dietary inclusion of 0.114% GAA increased LH and FSH. It can be concluded that GAA addition, as a supplement at the used levels, cannot be used for improving performance in laying hens receiving the corn-soybean meal based diets and it cannot appropriate source to sparing arginine at these levels in laying hens. The use of higher levels and also measuring creatine in muscle will be suggested in future studies for understanding relation GAA and creatine in laying hens.

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Research Article

The Role of Cuttlebone and Cuttlebone Derived Hydroxyapatite with Platelet Rich Plasma on Tibial Bone Defect Healing in Rabbit: An Experimental Study

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Abstract

Today marine-derived biologic scaffolds are popular due to their biocompatibility and high regenerative potential. Previous studies prepared hydroxyapatite from cuttlebone, the internal shell of cuttlefish; However, its biocompatibility and bioactivity has not been fully studied especially *in-vivo*. The aim of this study was to evaluate cuttlebone-derived hydroxyapatite in-vivo potential and possible synergistic effect of platelet rich plasma with this scaffold in promoting bone healing. Hydroxyapatite was prepared from cuttlebone *(Sepia officinalis)* via hydrothermal transformation. The conversion and microstructure of prepared material was assessed by scanning electron microscopy (SEM) and x-ray diffraction (XRD) analysis. Fifteen male white New Zealand rabbits were randomly divided to 6 groups each containing 5 limbs. In order to reduce sample size right and left pelvic limbs of rabbits were used as separate groups. Full thickness bi-cortical defects were created bilaterally in proximal tibia. The defect was left untreated in negative control group. In experimental group I to V the defect was filled with platelet rich plasma, raw cuttlebone, raw cuttlebone combined with platelet rich plasma, cuttlebone derived hydroxyapatite and cuttlebone derived hydroxyapatite combined with platelet rich plasma, respectively. Histopathological evaluation was performed on specimens received on day 56. Bone healing was assessed according to union, spongiosa, cortex and bone marrow indices. Our results demonstrated that Group I was superior to negative control group in defined indices. Groups IV and V showed preferable outcomes regarding to union and cortex indices in comparison to groups II and III. Also acceptable degree of spongiosa formation was observed in all groups. Cuttlebone derived hydroxyapatite could be an appropriate biomaterial to stimulate bone formation and enhance bone regeneration. Furthermore platelet rich plasma was successful in advancement of bone marrow formation.

Keywords: *Bone defect, Cuttlebone, Hydroxyapatite, Platelet rich plasma, Rabbit*

Tavşanlarda Mürekkepbalığı Kabuğu ve Kan Pulcuğundan Zengin Plazma İle Birlikte Mürekkep Balığı Kaynaklı Hidroksiapatitin Tibial Kemik Hasarının İyileştirilmesindeki Rolü: Deneysel Bir Çalışma

Özet

Günümüzde deniz ürünleri kaynaklı doku yapı iskeleleri, biyouyumlu ve yüksek yenilenme potansiyeline sahip olmaları nedeniyle popülerdirler. Mürekkep balığının iç kabuğu olan Mürekkepbalığı kabuğundan elde edilmiş hidroksiapatit ile yapılmış önceki çalışmalarda kabuğun biyouyumluluğu ve biyoaktivitesi özellikle in vivo olmak üzere tam olarak çalışılmamıştır. Bu çalışmanın amacı, mürekkep balığı kaynaklı hidroksiapatitin *in vivo* potansiyelini ve bu doku iskelesi ile kan pulcuğundan zengin plazmanın kemik iyileşmesini etkilemedeki sinerjik etkisini araştırmaktır. Hidroksiapatit mürekkepbalığı *(Sepia officinalis)* kabuğundan hidrotermal transformasyon ile elde edildi. Hazırlanan materyalin dönüştürme ve mikroyapısı Tarayıcı elektron mikroskop (SEM) ve x ışını kırımı (XRD) analizi ile kontrol edildi. On beş erkek beyaz Yeni Zelanda tavşanı rastgele olarak her birinde 5 bacak olacak şekilde toplam 6 gruba ayrıldı. Hayvan sayısını azaltmak adına sağ ve sol arka bacakları ayrı gruplar olarak kullanıldı. Proksimal tibiada bilateral olarak tüm katman biyokortikal hasar oluşturuldu. Negatif kontrol grubunda hasar tedavi edilmeksizin bırakıldı. Oluşturulan hasar I'den V'e kadar olan deneysel gruplarda sırasıyla kan pulcuğundan zengin plazma, işlenmemiş mürekkepbalığı kabuğu, işlenmemiş mürekkepbalığı kabuğu ile birlikte kan pulcuğundan zengin plazma, mürekkepbalığı kaynaklı hidroksiapatit ve mürekkepbalığı kaynaklı hidroksiapatit ile birlikte kan pulcuğundan zengin plazma ile dolduruldu. 56. günde alınan örneklerde histopatolojik inceleme gerçekleştirildi. Kemik iyileşmesi birleşme, spongioz, korteks ve kemik iliği belirteçleri göz önüne alınarak değerlendirildi. Elde edilen sonuçlar, göz önüne alınan belirteçler yönünden Grup I'in negatif kontrole göre daha üstün olduğunu gösterdi. Grup IV ve V'de grup II ve III ile karşılaştırıldığında birleşme ve korteks belirteçleri bakımından tercih edilebilir sonuçlar oluştuğu gözlemlendi. Tüm gruplarda kabul edilebilir düzeyde spongioz oluşumun gerçekleştiği gözlemlendi. Mürekkepbalığı kaynaklı hidroksiapatit kemik oluşumunu uyarmak ve kemik rejenerasyonunu geliştirmede uygun bir biyomateryal olabilir. Ayrıca kan pulcuğundan zengin plazmanın kemik iliği oluşumunu geliştirmede başarılı olduğu gözlemlendi.

Anahtar sözcükler: *Kemik hasarı, Mürekkepbalığı kabuğu, Hidroksiapatit, Kan pulcuğundan zengin plazma, Tavşan*

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INTRODUCTION

Bone is a dynamic tissue rich in blood vessels that acts as a structural and functional support in vertebrate's body. This specialized hard connective tissue undergoes remodeling and chemical exchange with other parts of body constantly [1-5]. The majority of bone injuries go through a gradual healing process without any scar formation and with considerable resumption of bone characteristics so that eventually the newly formed bony tissue is indistinguishable from peripheral healthy bone; nevertheless in extensive bone injuries like traumas, tumors and skeletal abnormalities healing is not successful because the extent of damage exceed the regenerative potential of bone. From past to now researchers have been looking for appropriate methods to potentiate and accelerate healing process or to replace the lost bone [3-8]. In different clinical conditions there are several therapeutic options available in order to reconstruct the defected bone; each of which has advantages and disadvantages [8,9]. Among these, bone grafts have been popular to researchers. Although autologous bone grafts have been mentioned as a gold standard, disadvantages of this method such as prolonged surgery time, pain and hemorrhage has limited its application lately [6,8,9]. Ideal bone substitutes should be non-immunogenic, bioactive, osteoconductive and osteoinductive, biodegradable, sterilizable, thermally non-conductive, traceable in vivo, readily available and economical [7-9]. Hydroxyapatite the main mineral component of hard tissues and one of the most stable forms of calcium phosphate has been utilized widely as a bone substitute in orthopedic and maxillofacial surgery in three past decades. It is a porous material that makes vascular ingrowth possible and provides oxygen and nutrients for cells [8-13]. Nowadays researchers are looking for natural materials considering them superior and more desirable [6,8,14]. Heretofore several raw materials such as eggshell and animal bone have been used to prepare hydroxyapatite; however, religious and social limitations along with disease transmission have made the use of these sources restricted [10,12,13,15]. Marine sources are novel in this field. Aquatic living organisms such as Coral, Nacre and Cuttlefish have been used in order to enhance bone regeneration recently [16,17]. Cuttlebone (CB) is the hard internal structure of cuttlefish consists of crystals of calcium carbonate (Aragonite) which plays essential role in protecting organism vital organs and act as a floating tank besides. It is biocompatible, osteoconductive and has plasticity in regard to morphology and mineral composition [10,13,18-20]. In addition, this structure is available in many oceans and seas worldwide with low expense. Parallel interconnected sheets give CB porosity. The pores diameter is in the range of 200-600 micrometer and therefore they are optimum for new bone formation and neovascularization [19,21]. The special structure and unique characteristics have encouraged researchers to try raw CB or cuttlebone derived hydroxyapatite (CBHA) in the field of tissue engineering and bone healing. Different methods have been used to prepare hydroxyapatite from CB recently which the most popular is hydrothermal synthesis. Time and temperature of the reaction are the main variables in this process [10,13,14,20,22,23]. Another biologic source that has widely introduced lately is platelet rich plasma (PRP); defined as a fraction of blood which platelet concentration is above baseline in it. PRP have been applied as an economical angiogenic orthobiologic in association with ceramics such as hydroxyapatite and majority of studies reported promising results; however some found opposite outcome so its regenerative capability is still controversial [24-27]. The aim of present study was to evaluate *in-vivo* effectiveness of raw CB and CBHA prepared by hydrothermal synthesis along with PRP in bone repair and to investigate probable synergism between these biomaterials.

MATERIAL and METHODS

Hydroxyapatite Preparation

CB was extracted from Cuttlefish (Sepia pharaonis from Persian Gulf), washed with distilled water and dried. The dorsal shield was removed and the lamellar part was cut into small blocks of about 1* 1*1 cm^2 by lancet and to remove organic residues immersed into 5% NaClO for 48 h. For hydrothermal synthesis the previously described method was slightly modified [22]. Briefly the required volume of 0.6 M aqueous solution of $NH_4H_2PO_4$ was added to yield a molar ratio of Ca/P = 1.67. The mixture was then sealed in a Teflon lined stainless steel pressure vessel and heated at 200°C in an electric furnace for 24 h. The resultant was dried under airflow prior to use. The raw CB was sterilized with Υ irradiation prior to *In vivo* implantation *(Fig. 1A-D)*.

Characterization of CB and CBHA

The microstructure and surface morphology of CB and CBHA were examined via SEM (SERON AIS-2100, Amirkabir University of technology, Tehran, Iran) after sputter coating with gold. To evaluate the composition of the raw and converted material, XRD analysis was performed. XRD patterns collected from 10-90° 2Θ at a 0.04°/min scanning rate.

PRP Preparation

After clipping and scrubbing the thoracic region adjacent to heart, the animals were anesthetized and positioned laterally and 8 mL fresh blood was obtained by intracardiac application of a long needle and transferred into Acid Citrate Dextrose containing sterile vacutainer immediately. The initial platelet count of blood samples were analyzed by veterinary automatic cell counter (NIHON KOHDEN, Japan) and the samples were centrifuged at 2400 rpm (SIGMA, Germany) for 20 min primarily in order to separate plasma. Separated plasma then centrifuged at 3600 rpm for 15 min once more. After this stage the supernatant plasma was

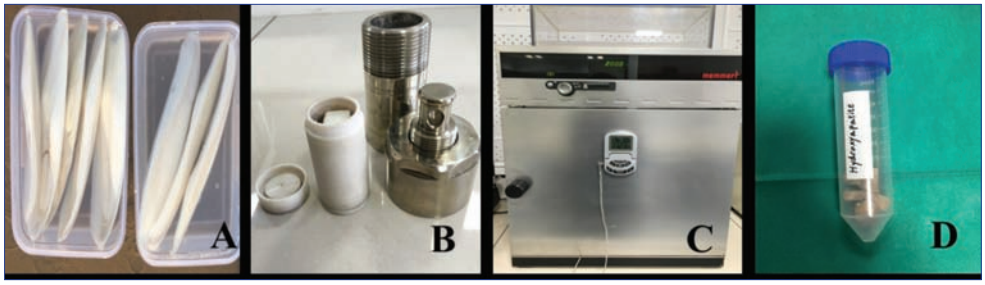


Fig 1. Hydroxyapatite preparation (A-D) A: cuttlebone extracted from cuttlefish, B: Teflon lined stainless steel pressure vessel, C: Electric furnace, D: Prepared hydroxyapatite

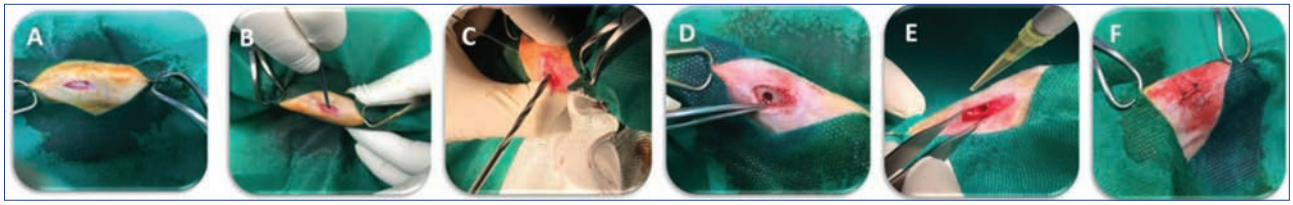


Fig 2. Surgical procedure A: Skin incision. B: Periosteal elevation and bone exposure. C and D: Tibial drill hole in order to make a defect. E: Platelet rich plasma application. F: Subcutaneous tissue and skin suture

removed and the remaining volume (about 0.5 mL) was collected and accounted as PRP. Finally the platelet counts were measured again and if the count was 3-5 times higher than the baseline count of the platelets, the samples were considered acceptable for the experiment.

In Vivo Testing, Fluoroscopy and Histopathological Examination

The animal research protocol was reviewed and approved by Iran society for the prevention of cruelty to animals (SPCA) and met Iranian Laboratory animals Ethic frameworks according to reference number IAEC 4-08/2. Fifteen adult male New Zealand white rabbits with similar average weight and age were kept under same environmental and nutritional conditions for two weeks for adaptation. Animals were randomly divided into 6 groups, including negative control group (untreated), group I (treated with PRP), group II (treated with raw CB), group III (treated with raw CB and PRP), group IV (treated with CBHA) and group IV (treated with CBHA and PRP). In order to involve fewer animals in the study we designed to use both legs of each animal; therefore, the animals left and right legs were clipped and scrubbed from the mid-shaft of the femur to tibia. Anesthesia was inducted in all groups with a combination of 40 mg/kg ketamine hydrochloride (10%®, Alfasan, Woerden, Netherlands) and 0.2 mg/kg medetomidine hydrochloride (Dorben Vet®, Spain SYVA s.a.u, Espain) and maintained with 3% Isoflurane. The animals were positioned in dorsal recumbency and the skin was incised in the proximal and medial region of the crus for about 2 cm *(Fig. 2A)*. After subcutaneous dissection, bone was exposed, the periosteum was pulled over *(Fig. 2B)* and a defect was created in tibia by 3.5 millimeter diameter slow speed orthopedic drill concomitant with irrigation in such a way that drill was entered from internal surface of the bone and moved out from the external surface *(Fig. 2C,D)*. In negative control group (left legs of 5 rabbits), the defect was left untreated, the periosteum was returned to its place and subcutaneous tissue and skin was sutured with continuous and interrupted suture patterns with 4-0 polyglactin 910 and 4-0 nylon, respectively *(Fig. 2F)*. In group I (right legs of same rabbits in negative control group), PRP was applied in the defect *(Fig. 2E)* after similar preparation and surgical procedure. CB was used as defect filler in group II (left legs of another 5 rabbits) and CB and PRP in group III (right legs of same rabbits). In group IV (left legs of another 5 rabbits) and v (right legs of same rabbits), CBHA and CBHA and PRP was implanted into the tibial bone defect respectively. Post-operative cares were included broad spectrum antibiotic and analgesic medications (5 mg/kg Enrofloxacine and 0.3 mg/kg meloxicam, Razak laboratory, Tehran, Iran) and adequate food and water intake. It should be noted that no considerable pain and discomfort was observed in any animal after surgery, because all animals were able to eat and drink adequately and bear weight. After 14 days the skin sutures were removed. Fluoroscopy imaging was done on day zero, immediately after surgery *(Fig. 3A,B)* to check bone integrity and on day 56 after surgery *(Fig. 3C,D)* in order to ensure the bone is unbroken and the implants are in place properly. The animals were euthanized 8 weeks after implantation and the defect sites along with the surrounding bone areas were dissected from the host bone. Specimens were put in 10% formalin buffer for fixation and a combination of 8% formic acid-8% hydrochloric acid for decalcification. After decalcification the specimens were dehydrated in a serial increasing concentration of ethanol, embedded in paraffin and sectioned. The sections were stained using hematoxylin and eosin and observed via light microscopy. Four indices (Union, Spongiosa, Cortex and Bone marrow) were defined for evaluation of bone healing according to Korkmaz et al.[28] study.

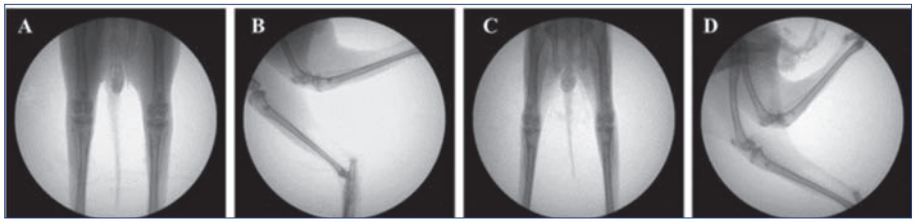


Fig 3. A and B: Fluoroscopic views of limbs on day zero. C and D: Fluoroscopic views of hind limbs on day 56

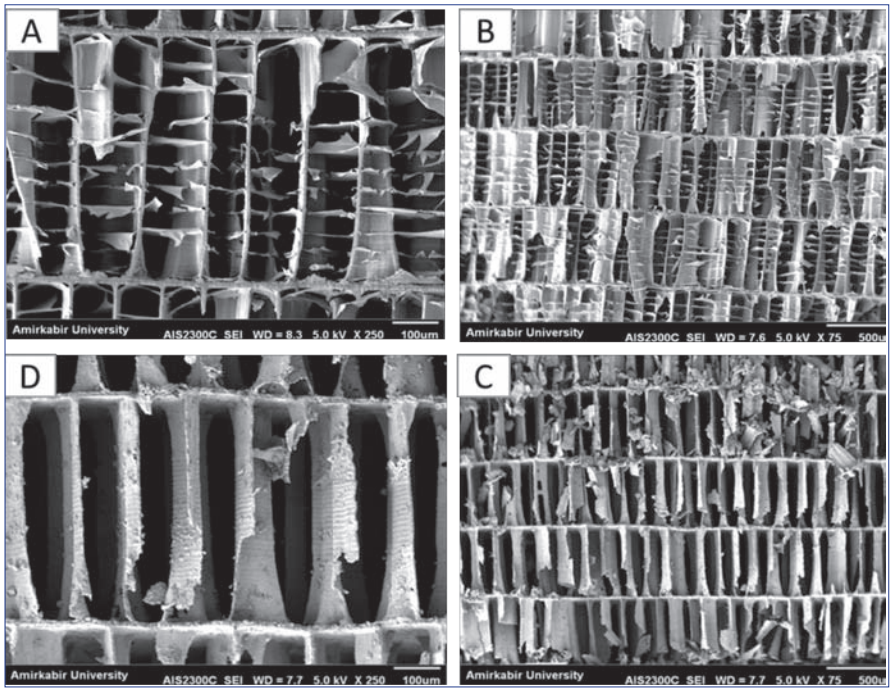


Fig 4. SEM image showing microstructure and surface characterization of raw cuttlebone (A, B) and hydrothermally converted hydroxyapatite (C, D); SEM, scanning electron microscopy

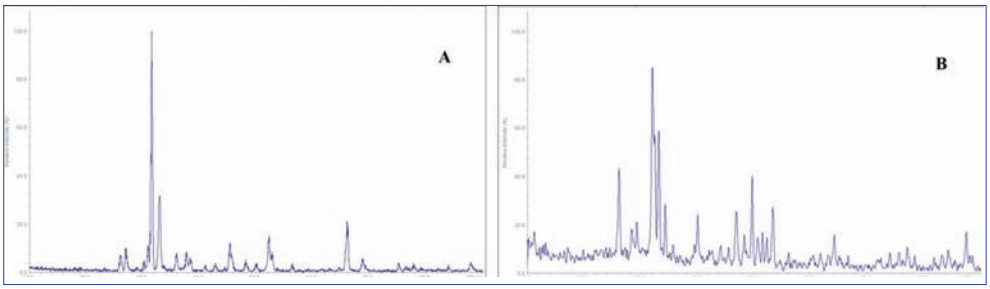


Fig 5. XRD pattern of raw cuttlebone (A) and hydrothermally converted hydroxyapatite (B).
XRD, x-ray diffraction

RESULTS

Characterization of CB and CBHA

The SEM image from raw CB demonstrated porous structure (pore size approximately 100 µm) with several interconnected chambers separated by vertical pillars. SEM micrograph of CBHA revealed similar structure; this indicates that most of the characteristic surface morphologies were preserved through hydrothermal synthesis process. *Fig. 4A,B* shows SEM micrographs of raw CB and *Fig. 4C,D* shows SEM micrographs of prepared CB. XRD patterns of CB before (A) and after (B) the conversion are shown in *Fig. 5*. XRD pattern of raw CB fairly resembled aragonite (crystal form of calcium carbonate). Conversion to hydroxyapatite was confirmed by XRD analysis. Close agreement was present between peeks of the specimen and the standard hydroxyapatite.

Histopathological Study

Each group received a score for defined indices *(Table 1)*. Group I (defect filled with PRP) was superior to negative control group in all of the investigated indices. Hydroxyapatite groups (IV and V) showed preferable outcomes regarding to union and cortex indices. Reorganized spongiosa formation and complete reorganized formation was identified in respect of spongiosa in all groups except negative control. PRP stimulated bone marrow formation according to results. In negative control group, the defect site was covered with abundant amount of fibrous connective tissue and some amount of cartilaginous callus

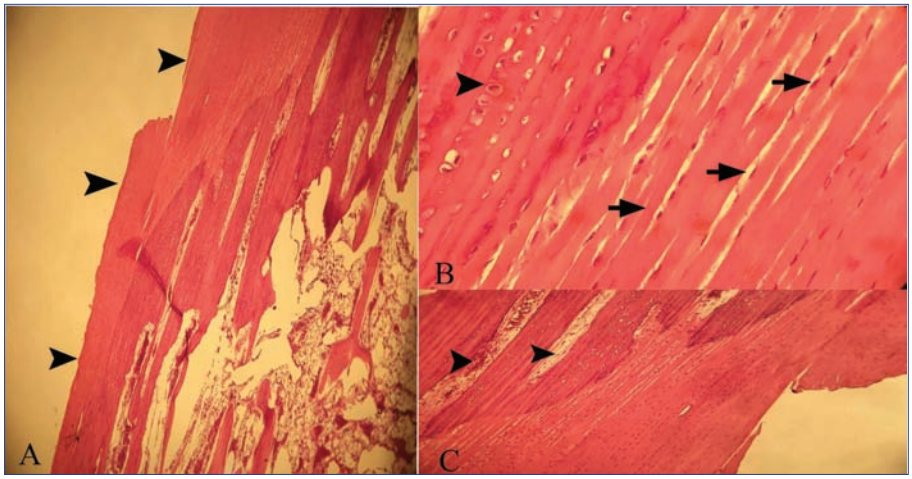


Fig 6. H&E stained images of defect site in negative control group on day 56. A: Considerable amount of fibrous connective tissue *(arrowhead)* and some amount of cartilaginous callus are visible in the defect site (×4). B: Fibrous connective tissue containing collagen fibers and fibrocyte nucleus *(arrow)* and cartilaginous callus containing chondrocytes *(arrowhead)* is evident (×40). C: Bone marrow cavities *(arrowhead)* (×10)

Table 1. *Histopathological results*

Index	Evaluation Scale	Score	Experimental Groups					
			Negative Control	Group I	Group II	Group III	Group IV	Group v
Union	No sign of union	0	1	2	3	3	4	4
	Fibrous union	1						
	Osteochondral union	2						
	Bone union	3						
	Complete reorganization	4						
Spongiosa	No sign of cellular activity	0	2	3	3	4	4	4
	Early bone formation	1						
	Active new bone formation	2						
	Reorganized spongiosa formation	3						
	Complete reorganized formation	4						
Cortex	Absence of cortex	0	2	3	3	3	4	4
	Early detection	1						
	Initiation of formation	2						
	Reorganization in majority	3						
	Complete organization	4						
Bone marrow	Not available	0	2	3	4	4	3	4
	Detection of fibrinous material	1						
	Defect occupying more than half	2						
	Fully occupying the red Bone marrow	3						
	Adult type fatty marrow	4						
Summation		16	7	11	13	14	15	16

was present. In addition collagen containing connective tissue and chondrocyte containing callus were present along with scant cancellous tissue *(Fig. 6)*. In group I, the defect site was enveloped with osteocartilaginous callus and chondrocytes and osteocytes were present. Bone marrow cavities were filled with hematopoietic bone marrow *(Fig. 7)*. In groups II and III, a thin osseous tissue had coated the defect site and inflammatory reaction was seen in the region of bone marrow. Also inflammatory response characterized by mononuclear phagocytic cells in the periphery of CB remnants was detected *(Fig. 8, Fig. 9)*. In group IV, new bone formation was determined at the defect site. Furthermore blood was noticed in bone marrow cavities concomitant with hydroxyapatite residues *(Fig. 10)*. In the last group (V), formation of Haversian systems was seen and hydroxyapatite residues were observed in bone marrow cavities *(Fig. 11)*.

Fluoroscopy

Proximal tibia was broken in one rabbit in group II on

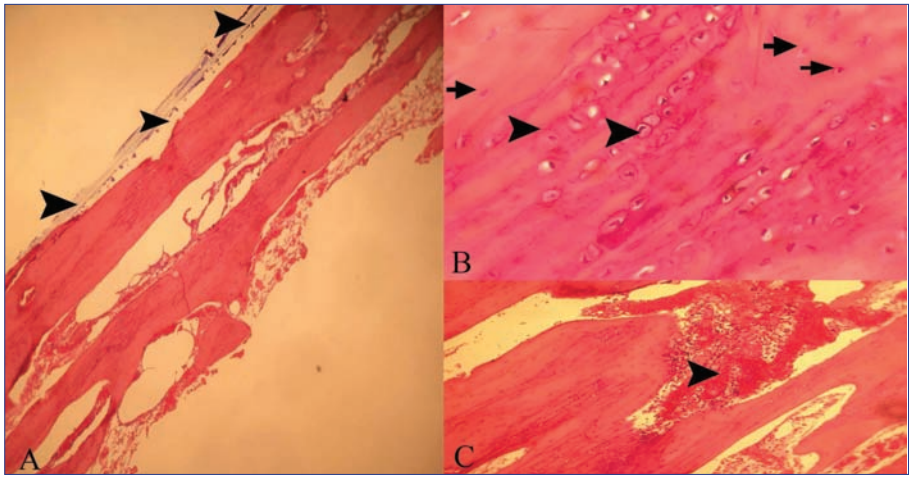
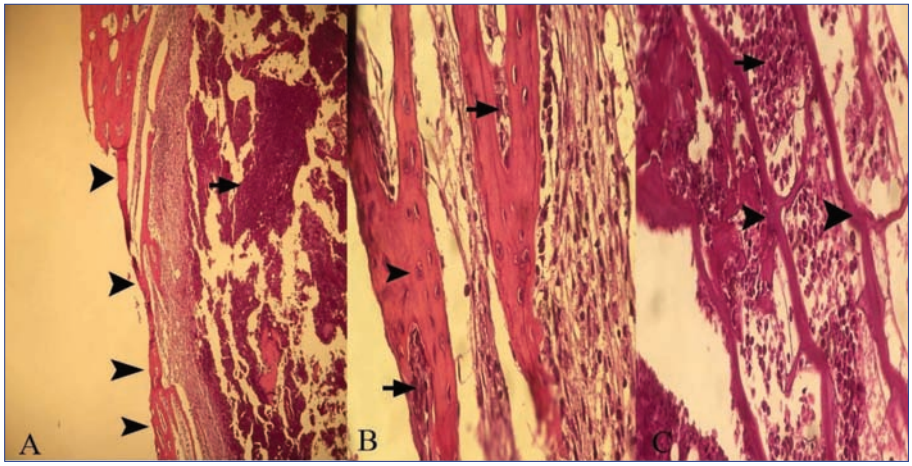


Fig 7. H&E stained images of defect site in group I on day 56. A: The defect site is covered by a large quantity of osteo-cartilaginous callus *(arrowhead)* (×4). B: Chondrocytes *(arrowhead)* and Osteocytes *(arrow)* are visible at the healing site (×40). C: Hematopoietic bone marrow *(arrowhead)* has filled bone marrow cavities (×10)

Fig 8. H&E stained images of the defect site in group II on day 56. A: Arrowheads indicate a thin covering of osseous tissue and the arrow indicates inflammatory reaction in the bone marrow region (×4). B: Endosteal osteoblasts and lacunar hypertrophic osteocytes are evident (×40). C: Cuttlebone remnants *(arrowhead)* surrounded by mononuclear inflammatory response (×40)



fluoroscopy imaging done on day zero, after surgery *(Fig. 12,A)*. Fixation by intramedullary pin was done *(Fig. 12,B)*. Fifty six days after surgery, union was achieved *(Fig. 12,C)* and the pin was removed after euthanasia. In the other groups the bone integrity was preserved, no fractures were seen and all implants were in their place *(Fig. 3)*.

DISCUSSION

Nowadays bone substitutes have opened new insights in bone regeneration by reducing the limitations of bone graft such as low availability, high cost, zoonotic diseases transmission, immune reactions and so on [4,7,8]. In general natural and synthetic scaffolds are available; each has specific advantages and disadvantages [6,8]. Biocompatibility, biodegradation and regenerative characteristics including osteoinduction, osteoconduction and osteogenesis are the most important advantages of natural scaffolds over synthetic ones [8]. Hydroxyapatite is well-known because of high similarity to mineral bone composition [8-10,29]. Some natural sources have capacity to be converted into hydroxyapatite, for example Ni et al.[30] obtained hydroxyapatite from nacre, and Gao et al.[31] obtained it from coral. In addition different species of wood were pyrolized and its carbonate residues saturated with calcium salts to achieve bioactive hydroxyapatite [9]. CB is a unique marine biomaterial potentially convertable to hydroxyapatite that has attracted attention of researchers currently [10,13,14,19,20,22]. CB compatibility with bone mineral composition was demonstrated by quantitative analysis of sodium, magnesium, pottasium and calcium ions in CB and human elbow bone [18,19]. Many researches focused on evaluation of CB effect on bone regeneration, alone or in combination with grafts, growth factors and cell culture [10,18,19,29,32,33]. For instance CB mechanical and biological characteristics as an bioactive acrylic bone cement was assessed previously *in vivo* and efficient implant integration with bone was reported and there was no evidence of secondary infection [18]. In another study, Kim [20] and colleagues investigated attachment and differentiation of mesenchymal cells on CB dorsal shield and lamellar part and concluded that lamellar matrix permits better cellular penetration than dorsal shield, although its a brittle structure and needs more investigations in order to increase strength. Yi [32] and colleagues showed synergistic effect of CB, autologous bone marrow and sodium hyaloronate in healing of radial bone defect in rabbit. Liu et al.[33] confirmed the positive effect of BMP impregnated CB on osteogenesis and neovascularization of rat calvarial defect by histopathological examination.

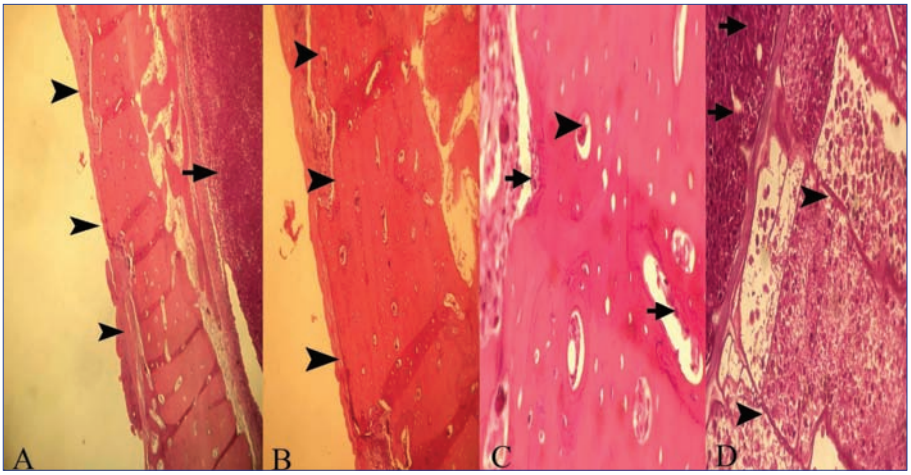
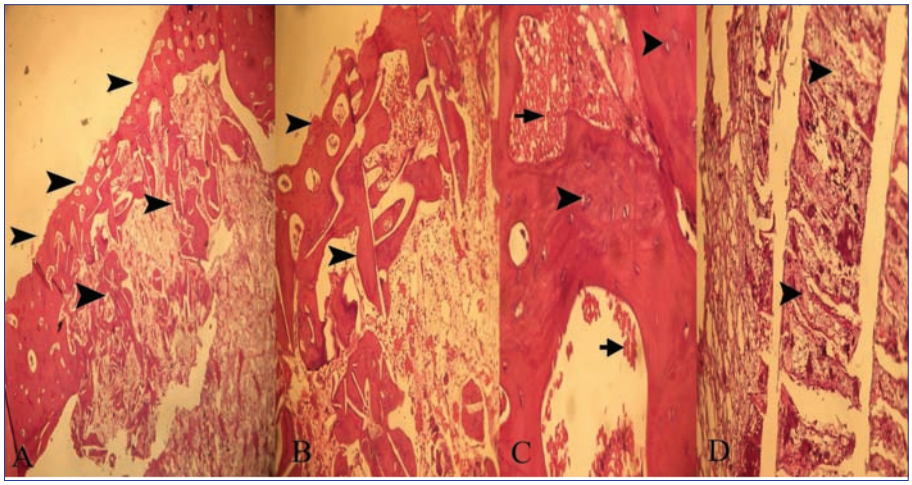


Fig 9. H&E stained images of defect site in group III on day 56. A: Marrow derived inflammatory reaction *(arrow)* and osseous tissue *(arrowhead)* are observable at the defect site (×4). B: *Arrowheads* indicate osseous callus at the healing site (×40). C: Osteoblasts *(arrow)* are noticeable in the endosteum and bone marrow cavities (×40). D: Cuttlebone remnants are encompassed with mononuclear inflammatory response *(arrow)* (×40)

Fig 10. H&E stained images of defect site in group IV on day 56. A: Arrowhead demonstrates osseous tissue at defect site (×40). B: New bone formation *(arrowhead)* is indicated (×40). C: Blood-filled bone marrow cavities *(arrow)* and osteocytes *(arrowhead)* are visible (×40). D: Hydroxyapatite residues are present in bone marrow cavities (×40)



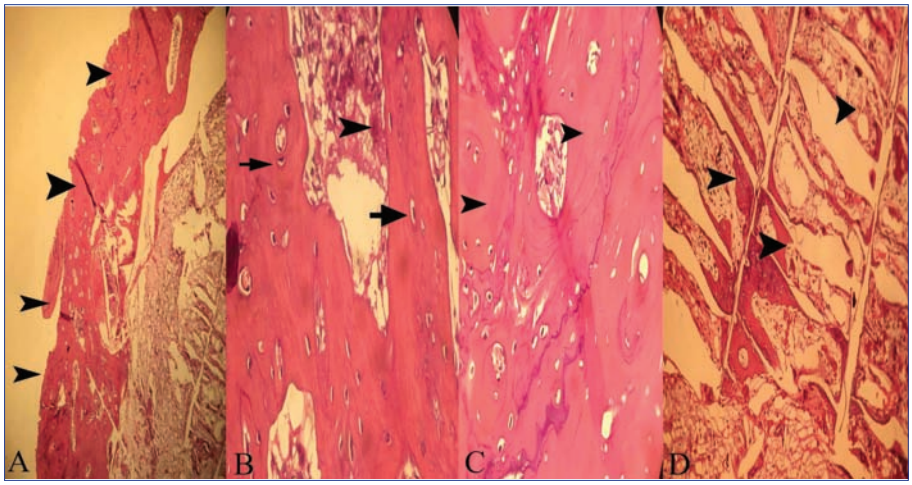


Fig 11. H&E stained images of defect site in group V on day 56. A: Osseous tissue *(arrowhead)* had covered the defect site (×4). B: *Arrows* are indicative of osteoblasts in the endosteum and *arrowhead* is indicative of hypertrophic osteocytes in the lacuna (×40). C: Haversian systems *(arrowhead)* are observable in newly formed osseous tissue (×40). D: Hydroxyapatite remnants *(arrowhead)* are visible in bone marrow cavities (×40)

In similar clinical setting, Dogan and Okamus [19] compared raw CB, demineralized bone matrix, bovine cancellous bone and tricalcium phosphate in regeneration of metaphyseal radial bone defect in rabbit. CB was inferior to other groups radiologically but it presented the best result in histopathological evaluations. In our study fracture of proximal tibia was likely due to excessive pressure at the site of drilling and being close to bone cortex. However, fracture was observed only in one rabbit of experimental group II. According to fluoroscopic views on day 56 *(Fig. 12 C)*, fracture line of proximal tibia in mentioned rabbit was disappeared and healing was achieved. Since CB has many positive characteristics and is readily available in our country by low expence we used it as a biomaterial in this study. CB showed promissive effects on bone healing purely and also incombination with PRP, however it's effect

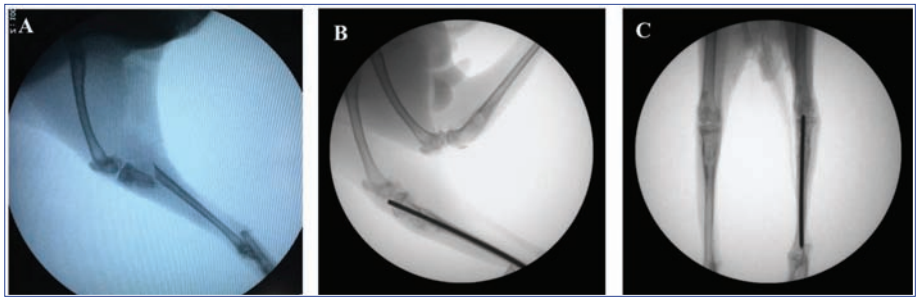


Fig 12. Fluoroscopic views of broken limb of the rabbit in group II. A: Immediately after surgery, B: Fixation of the fracture by intramedullary pinning, C: Union on day 56

was inferior in comparison to CBHA based on our histopathological results *(Table 1)*. Other studies utilized CB after processing it into hydroxyapatite [10,13,14,22,23]. Rocha et al.[21] produced hydroxyapatite from CB by hydrothermal synthesis for the first time in literature review. Ivankovic et al.[22] synthetized hydroxyapatite in the temperature range of 140-220ºC for various times from 20 min to 48 h in order to provide a suitable scaffold for tissue engineering. They achieved complete conversion of aragonite into hydroxyapatite in 200ºC and 24 h. In present study hydroxyapatite blocks were prepared via hydrothermal synthesis in 200ºC, within 24 h in electric furnace and conversion was confirmed by XRD analysis *(Fig. 5)*. Porosity and the pores size are responsible for providing optimum environment for tissue regeneration and nutrient diffusion between cells and the neighboring area. These characteristics depend on the consumed material and the fabrication method extensively [3,6,8]. The pores provide supportive structure for cell growth, neovascularization and development of bone tissue [10]. Too small pores will be obstructed by cells and this will preclude the afformentioned processes; while big pores will decrease mechanichal strength beside establishing better interaction with the surrounding tissue [8]. It was reported that 100-400 micrometer pore size is ideal although 200-350 µm was reported also elsewhere [8-10]. CB is a porous material with the pore size between 200-600 µm and therefore its suitable for new bone formation and neovascularization [10,11,14,21]. In our study the pore size was measured approximately 100 µm. Initial porous interconnected structure was preserved under hydrothermal procedure according to SEM images *(Fig. 4)*. Acceptable results were reported from *in vitro* application of CBHA [10,14,34]. Buttistella et al.[34] evaluated osteogenic markers expression and MC3T3-E1 cells proliferation on CBHA. In another study Kim et al.[10] designed a scaffold by adding polycaprolactone to CBHA. The scaffold induced efficient cell response and showed amazing potential for application in tissue engineering. According to Hongmin et al.[14], CBHA supported human mesenchymal cell growth and was appropriate for filling bone defects. Although CB bioactivity *in vitro* has been showed, CB biocompatibility *in vivo* has not been fully studied. Hongmin et al.[14] implanted mice dorsal subcutaneous pockets with CB and CBHA and reported ectopic bone formation just in hydroxyapatite group. In contrast in present study, although hydroxyapatite showed superior bioactivity and regenerative properties, the effectiveness of pure CB on healing process could'nt be ignored. Kim et al.[10] compared *In vivo* bioactivity of CBHA and synthetic hydroxyapatite in rabbit calvarial bone deffect and higher bone formation and accelerated healing process was present in CBHA group [11]. In this study we evaluated CBHA in load axis of tibia. Histopathological examination showed that CBHA could induce new bone formation as a bone substitute in rabbit tibial bone defect. PRP, an inexpensive source rich in growth factors has emerged as an adjuvant therapy that may accelerate regenerative process recently [24-27]. Numerous experimental studies have applied PRP in association with bone grafts and substitutes; only some were successful [24,27,35]. Therefore despite PRP pervasive application, its clinical efficacy is yet questionable. The combination of hydroxyapatite and PRP was significantly superior versus 0.9% sterile salt solution and PRP in reconstruction of periodontal membrane lesions [27]. It is reported that human PRP combined with persian gulf coral can promote healing of rabbit radial bone defect [8]. A combination of autologous bone and PRP for alveolar cleft reconstruction was evaluated and no significant statistical difference was found between the two groups [27]. Because the effect of CBHA and PRP combination has not been investigated heretofore and also due to controversial regenerative potential of PRP we decided to assess PRP in experimental setting once more. PRP was applied concurrently by the time of implantation of CB and CBHA and on our histopathological studies demonstrated that PRP is capable of promoting bone marrow formation and development during healing process. In present study the regenerative potential and biocompatibility of CB and CBHA was compared via introduction of these two material into rabbit tibial bone defect. We concluded that although raw CB itself is an admissible bone defect filler, the prepared hydroxyapatite is more promisive according to defined histopathologic indices. We introduced raw and processed CB as an accetable marine source that can induce new bone formation, but still further studies with farther number of animal models and longer evaluation period are required in order to assess this biomaterial precisely and follow the remodeling phase of the bone healing process.

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Conflict of Interest

None.

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Research Article

Investigation of Changes in Metabolic Parameters and Paraoxonase-1 During the Transition Period in Turkish Saanen Goats [1]

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Abstract

The aim of this study was to evaluate the metabolic changes and PON-1 levels in Turkish Saanen goats during the transition period. In the study, 60 Turkish Saanen goats were used and blood samples were taken from the jugular vein on days -30, -15, 0, +15, and +30 from parturition. In order to monitor metabolic profile changes, alkaline phosphatase (ALP), cholesterol (CHO), glucose (GLU), calcium (CA), phosphorus (PHO), total protein (TOP), triglyceride (TG), paraoxonase-1 (PON-1), β-hydroxybutyric acid (BHBA) and non-esterified fatty acid (NEFA) levels were determined. Serum alkaline phosphatase levels were lower on days -30, 0 and +15, and higher on day +30 (P<0.001). Serum cholesterol and total protein levels were lower on days -30 and 0, and higher on day +30 (P<0.001). Serum levels of glucose, calcium and phosphorus were lower on day 0, and higher on day +30 (P<0.001). Serum triglyceride levels were lower on days -15 and 0, and higher on days -30 and +30 (P<0.05). Serum paraoxonase-1 levels were lower on day 0, and higher on day -30 (P<0.05). Serum β-hydroxybutyric acid levels were lower on day -30, and higher on day +15 (P<0.05). Serum non-esterified fatty acid levels were lower on days -30 and +30, and were higher on days -15 and 0 (P<0.001). In conclusion, the results of our study showed that clear changes in levels of metabolic parameters and PON-1 were seen during the transition period in Turkish Saanen goats.

Keywords: *Metabolic parameter, PON-1, Transition period, Turkish Saanen goat*

Türk Saanen Keçilerinde Geçiş Dönemi Boyunca Metabolik Parametreler ve Paraoksonaz-1 Değişiminin İncelenmesi

Özet

Bu çalışmanın amacı, Türk Saanen keçilerinde geçiş dönemi boyunca metabolik parametreler ve paraoksonaz-1 düzeylerindeki değişimi incelenmekti. Çalışmada 60 adet Türk Saanen keçisi ve bu keçilerin juguler venasından doğumdan 30 ve 15 gün önce, doğumda ve doğumdan 15 ve 30 gün sonra alınan kan örnekleri kullanıldı. Metabolik profilde meydana gelen değişimleri belirlemek için alkalen fosfataz (ALP), kolesterol (CHO), glukoz (GLU), kalsiyum (CA), fosfor (PHO), total protein (TOP), trigliserit (TG), paraoksonaz-1 (PON-1), β-hidroksibütirik asit (BHBA) ve non-esterifiye yağ asidi (NEFA) düzeyleri belirlendi. Serum alkalen fosfataz düzeyleri -30, 0 ve +15. günlerde en düşük, +30. günde en yüksekti (P<0.001). Serum kolesterol ve total protein düzeyleri -30 ve 0. günlerde en düşük, +30. günde en yüksekti (P<0.001). Serum glukoz, kalsiyum ve fosfor düzeyleri 0. günde en düşük, +30. günde en yüksekti (P<0.001). Serum trigliserit düzeyleri -15 ve 0. günlerde en düşük, -30 ve +30. günde en yüksekti (P<0.05). Serum paraoksonaz-1 düzeyleri 0. günde en düşük, -30. günde en yüksekti (P<0.05). Serum β-hidroksibütirik asit düzeyleri -30. günde en düşük, +15. günde en yüksekti (P<0.05). Serum non-esterifiye yağ asidi düzeyleri -30 ve +30. günlerde en düşük, -15 ve 0. günlerde en yüksekti (P<0.001). Sonuç olarak, Türk Saanen keçilerinde geçiş dönemi boyunca metabolic parametreler ve paraoksonaz-1 düzeylerinde belirgin değişimlerin meydana geldiği belirlendi.

Anahtar sözcükler: *Geçiş dönemi, Metabolik parametre, PON-1, Türk Saanen keçisi*

INTRODUCTION

Turkish Saanen goat originated by crossing of Saanen breed and is commonly used in goat husbandry in Turkey according to higher milk production and fertility efficiency [1]. The average lactation duration and yield of Turkish Saanen goat are between 270-290 days and 500-600 kg (can reach up to 805 kg), respectively [1,2].

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Transition period defines the 3-week period before and after the parturition and it is mostly used for dairy cows. Important hormonal and metabolic changes occur during the transition period of cows. Immunosuppression, infections such as metritis, mastitis and fertility problems can be observed depending on these changes [3]. The transition period is the most critical period among lactation periods in terms of health, fertility, milk yield and thus the profit, especially in high-yielding animals [4].

Nutritional requirements for milk production and fetal growth prominently increase in the late pregnancy and early lactation period [4,5]. The energy requirement also increases in line with the rapidly increasing milk yield immediately after the calving. However, the feed consumption of cows is unable to meet their energy needs and thus negative energy balance (NED) occurs [4,6,7]. Negative energy balance is characterized with the fat tissue mobilization and rise in blood ketone bodies in order to meet energy requirements [7]. Immune system functions are negatively affected by this metabolic stress condition [8]. Physiological stress and an increase in oxygen requirements and energy demands occur depending on the onset of milk production and physiological changes in mammary glands. Oxidative stress occurs as a result of the excess accumulation of reactive oxygen species (ROS) due to the rise in oxygen consumptions [9,10]. There are metabolic changes in addition to the endocrine profile changes in goats in the periparturient period [11]. Even though lipid mobilizations [12] and oxidative stress [13,14] are specified in goats as in the cows, there are limited numbers of studies on the metabolic changes in high-yielding dairy goats.

Paraoxonase-1 is synthesized in liver and it is an enzyme associated with high-density lipoprotein (HDL) [15] and it is also specified as the negative acute phase protein [16]. PON-1 is used as oxidative stress biomarkers due to their protective roles on HDL and low density lipoproteins (LDL) [17]. According to the literature, even though there are studies about PON-1 in cattle [18-22], dogs [15,23,24] and horses [25], there is no study conducted on this enzyme in any goat breed.

To our knowledge, there are no published paper on changes of metabolic parameters during the transition period in Turkish Saanen does. We hypothesized that important changes at levels of metabolic parameters and PON-1 can occur during the transition period in Turkish Saanen goats. For this, we aimed to evaluate the metabolic changes in Turkish Saanen goats does during the transition period.

MATERIAL and METHODS

Animal, Housing and Management

The study was conducted on a commercial farm in Ankara, Turkey (39°53'E; 32°45'N) in the period from January to March 2014. Sixty Turkish Saanen goats (75% Saanen × 25% Kilis (B_1) crossbreed), aged 2 to 4 years, clinical healthy, with similar body weight (range of body weight was 50-55 kg) and body condition score (BCS of 3.0) [26] were included in the study. The goats with disorders related to calving or any metabolic and infectious diseases during study period were excluded from the study. All animals were housed in an outdoor paddock under natural conditions. The animals were fed with wheat straw, alfalfa hay and commercial compound (feed regimen (per doe) during dry period; 500 g commercial compound + 500 g alfalfa hay and *ad libitum* wheat straw; feed regimen (per doe) during early lactation period: 1000 g commercial compound + 1000 g alfalfa hay and ad libitum wheat straw). All animals had unlimited access to fresh water and mineralized salt blocks. In the farm, all animals were regularly vaccinated against brucellosis, peste des petits ruminants and foot and mouth disease and were routinely received worming treatment. After the parturition, kids were kept together with their does for suckling during 45 days in milk. After the suckling, all animals were milked once a day using a milking machine in the morning and the average milk yield was about 3 liter/day per doe. The average litter size at birth was 1.97±0.04 per doe (mean±SE).

Study Design, Blood Sampling and Biochemical Analyses

All does were subjected to controlled mating with Turkish Saanen bucks on natural oestrus. To determine pregnant does, transrectal ultrasonography was done using 7.5 megahertz (MHz) linear probe connected to a portable B-mode scanner (Hasvet 838®, Hasvet, Turkey) by the same observer up to 25 to 30 days after mating. Expected delivery dates were determined for each doe according to pregnancy diagnosis for blood sampling.

In the study, blood samples were collected from the jugular vein into Vacutainer tubes containing clot activator 4 h after the feeding on days of -30, -15, 0, +15 and +30 from expected parturition. These blood samples were centrifuged at 3.000 rpm for 20 min at 4°C within 1 h after the sampling and serum were kept in a freezer at −20°C until biochemical analyses were performed.

Levels of alkaline phosphatase (ALP), cholesterol (CHO), glucose (GLU), calcium (CA), phosphorus (PHO), total protein (TOP), triglyceride (TG), β-hydroxybutyric acid (BHBA), non-esterified fatty acid (NEFA) and paraoxonase-1 (PON-1) were measured in the samples. All the parameters except paraoxanase activity were measured using Erba XL 600 autoanalyzer and its accompanied commercial reagents (Erba-Diagnostics, Mannheim). Serum paraoxonase activitity was measured in a 37°C ambient temperature as the rate of hydrolysis of paraoxone at 412 nm in 0.05 mmol/L glycine buffer (pH 10.5) with 1 mmol/L $CaCl_2$. All biochemical analyses were conducted on Biochemistry Laboratory at Ankara University, Faculty of Veterinary Medicine.

The experimental procedures were approved by the Local

Ethical Committee on Animal Experiments of Ankara University in Ankara (Turkey) (Approval no; 2013-14-106).

Statistical Analyses

Before analyses, data were checked for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively. General linear model (GLM) for repeated measures procedure was used to evaluate the differences of blood serum metabolite parameters between period. A multiple comparison test of least-squares means was performed using the Bonferroni correction. P<0.05 was used as the criterion for significance. All results were presented as the means ± standard error of means (mean ± SEM). All data analysis was carried out by using the SPSS 14.01 (Licence No: 98692604) statistical program.

RESULTS

Serum ALP levels were lower on days -30, 0 and +15, and were higher on day +30 (P<0.001). Serum CHO and TOP levels were lower on days -30 and 0, and were higher on day +30 (P<0.001). Serum levels of GLU, CA and PHO were lower on day 0, and were higher on day +30 (P<0.001). Serum TG levels were lower on days -15 and 0, and were higher on days -30 and +30 (P<0.05). Serum PON-1 levels were lower on day 0, and were higher on day -30 (P<0.05). Serum BHBA levels were lower on day -30, and were higher on day +15 (P<0.05). Serum NEFA levels were lower on days -30 and +30, and were higher on days -15 and 0 (P<0.001). All results were presented in *Table 1* and *Table 2*.

DISCUSSION

In our study, serum ALP levels were similar in -30, -15, 0, and +15 days whereas it reached to its maximum amounts on the +30 day. ALP is an enzyme which is defined as the physical stress indicator [27]. Hepatic diseases, intestinal disorders, drug use, pregnancy, and various diseases are the factors which increase serum ALP levels [28]. Bogin et al.[29] stated that serum ALP levels prominently increased in cows with fatty liver. The maximum serum ALP levels on the +30 day can be related to the effect of the hyperketonemia on the fatty liver (it was detected that BHBA levels prominently increased in the postpartum period). In case of the negative energy balance, the glucose need is met by lipolysis by using body fat stores. In case fatty acids excessively accumulated in the liver, the liver functions are deteriorated and hepatic lipidosis occurs [30,31].

We detected that the maximum levels of TG on the 30 d postpartum and this finding can be supported with serum ALP outcome. The fat accumulation in the liver of cows with fatty liver syndrome occurs primarily as triglycerides [32]. Kalaitzakis et al.[33] specified that TG levels were high in cows died due to the fatty liver syndrome. They further claimed that the high TG levels were associated with high liver damage and poor prognosis.

Serum TOP levels were lower on the 30 d prepartum and 0 day whereas it was gradually increased and reached the higher levels on 30 d postpartum. This can be associated with the onset of colostrum production. Transition of immunoglobulins and serum proteins from blood to colostrum occurs with the onset of colostrum production in udder at prepartum period. These findings were in line with results of Sadjadian et al.[11] obtained from Saanen goats.

Cholesterol levels in goats are changeable depending on nutritional and physiological (pregnancy, lactation) conditions [34]. The serum CHO level was higher on the 15 d prepartum whereas it was in its minimum level on

Table 2. *Mean values* (± SEM) of PON-1 and keton *bodies in Turkish Saanen goats during the preparturient period*

Days	Paraoxonase-1	β-hydroxybutyric Acid	Non-esterified Fatty Acid
-30	168.30±9.05 ^{a}	0.62±0.04 ^{b}	0.30±0.03 ^{b}
-15	164.37±8.72 ^{a}	0.70±0.04 ^{ab}	0.54±0.05 ^{a}
0	138.43±7.02 ^{b}	0.73±0.06 ^{ab}	0.53±0.07 ^{a}
+15	161.17± 7.53 ^{a}	0.85±0.05 ^{a}	0.40±0.04 ^{ab}
+30	151.33±7.20 ^{ab}	0.70±0.06 ^{ab}	0.23±0.02 ^{b}
p	*	*	**

-30: *Prepartum 30;* ***-15:*** *Prepartum 15;* ***0:*** *Parturition;* ***+15:*** *Postpartum 15;* ***+30:*** *Postpartum 30; ** P<0.001; * P<0.05; ^{abc} The same superscript letters in the same column indicate no significant differences statistically*

Table 1. *Mean values (±SEM) of metabolic parameters and macrominerals in Turkish Saanen goats during the preparturient period*

Days	Alkaline Phosphatase	Cholesterol	Glucose	Calcium	Phosphorus	Total protein	Triglyceride
-30	27.77±3.57 ^{b}	65.78±2.92 ^{bc}	47.50±2.18 ^{b}	4.68±0.27 ^{b}	4.95±0.27 ^{b}	3.96±0.35 ^{bc}	88.94±2.52 ^{ab}
-15	33.50±3.81 ^{b}	76.06±3.40 ^{b}	50.78±3.89 ^{b}	5.06±0.30 ^{b}	5.04±0.26 ^{b}	5.19±0.36 ^{b}	80.10±1.96 ^{c}
0	23.77±5.53 ^{b}	59.24±2.50 ^{c}	33.18±2.39 ^{c}	3.54±0.22 ^{c}	4.89±0.34 ^{b}	3.24±0.27 ^{c}	80.60±2.24 ^{bc}
+15	29.05±4.30 ^{b}	74.60±2.85 ^{b}	48.22±2.12 ^{b}	5.30±0.21 ^{b}	5.88±0.39 ^{b}	5.08±0.29 ^{b}	85.04±2.32 ^{abc}
+30	59.05±7.90 ^{a}	108.86±2.99 ^{a}	67.93±1.81 ^{a}	7.96±0.14 ^{a}	9.44±0.35 ^{a}	8.62±0.16 ^{a}	90.33±2.03 ^{a}
P	**	**	**	**	**	**	*

-30: *Prepartum 30;* ***-15:*** *Prepartum 15;* ***0:*** *Parturition;* ***+15:*** *Postpartum 15;* ***+30:*** *Postpartum 30; ** P<0.001; * P<0.05; ^{abc} The same superscript letters in the same column indicate no significant differences statistically*

the 0 day. Higher serum CHO level was determined on the 30 d postpartum as a result of the gradual increase after kidding. These findings were in line with results of Sadjadian et al.[11] obtained from Saanen goats. According to results of previous study [11], stated that the low CHO levels in the last week of the pregnancy can be associated with the rise of needs for the fetal growth and increase in the steroid hormone synthesis [35]. It was also stated that the increase of CHO in the lactation period can also be related to the lipid immobilization [36]. Additionally, it can also be associated with prepartum and postpartum nutritional differences because animals were fed with energy rich feeds after the kidding compared to the dry period.

Serum glucose levels were similar on 30 d and 15 d prepartum. Even though we observed the minimum serum glucose level at the time of the kidding, it gradually increased and became maximum on the 30 d postpartum. Similarly, Sadjadian et al.[11] and Radin et al.[37] reported that serum glucose levels were higher in the early lactation period compared to prepartal period. This was associated with the decrease in the severity of the negative energy balance that was observed after the 15 d postpartum. This thought is verified with the rise in the BHBA levels on 15 d postpartum and the reduction of BHBA levels on 30 d postpartum day. Sadjadian et al.[11] detected that glucose levels were minimum in the two weeks before from kidding and it reached its peak level during the parturition. The researchers claim that endocrine changes during the parturition can be associated with the induction of the gluconeogenesis. In our study, we detected that serum glucose level was the lowest on the day of parturition. The decline may be associated with decreased food intake due to physiological stress of parturition. Another possible explanation is that this finding can be associated with the high incidence of the pregnancy toxemia (when evaluate the both glucose and BHBA levels together) which can occur due to the nutritional deficiencies.

Our results showed that serum levels of CA were lower on the 0 day and higher on day +30. Similarly, PHO were lower on the 0 day, and were higher on day +30 in serum samples. Ivanov et al.[38] determined that calcium need increased with the initiation of milk production and serum calcium levels decreased in dairy cows. Similarly, we also observed these changes in Turkish Saanen goats. Ca and P levels in goats in the late pregnancy and early lactation periods are associated with the Ca and P levels obtained by rations and Ca/P homeostasis [39]. We detected minimum levels of these parameters at kidding and they gradually reached their maximum levels on the 30 d postpartum. These findings can be associated with nutritional changes after the kidding. The amount of alfalfa hay that is a Ca-rich source is prominently increased in the postpartum period.

PON-1 is synthesized in the liver and it is a Ca-dependent and HDL associated enzyme [40]. PON-1 decreases the oxidative stress in transgenic mice [41], and there is an increase in the gene expressions related with oxidative stress in PON-1-knockout mice. Therefore, PON-1 is defined as the negative acute phase protein [16]. In our study, it was found that serum PON-1 levels were minimum on 0 day (138.43±7.02 U/ml) and maximum on -30 day (168.30±9.05 U/mL; P<0.05). Furthermore, PON-1 levels of -15 (164.37± 8.72 U/mL) and +15 (161.17± .53 U/mL) day were similar with levels of -30 day (168.30±9.05 U/mL). According to the changes in the PON-1 levels, it is possible to claim that the minimum oxidative stress level was detected on -30 day, it reaches its maximum level at kidding, and oxidative stress continues during the late pregnancy and early lactation period. Similarly, Turk et al.[18] reported that PON-1 level is lower in late pregnancy and early lactation in cows compared to other periods.

NEFAs are precursors of ketone bodies and reflect the mobilization from fatty storage. Fatty tissues are mobilized when glucose supply cannot meet requirements for pregnancy and milk production. This condition results in a decrease and an increase of glucose and NEFA concentrations in blood stream, respectively [42]. Serum NEFA levels were lower on days -30 (0.30±0.03 mmol/L) and +30 (0.23±0.02 mmol/L), and were higher on days -15 (0.54±0.05 mmol/L) and 0 (0.53±0.07 mmol/L; P<0.001). The higher levels of NEFA from 15 d prepartum to parturition can be supported by serum glucose outcome. Serum NEFA threshold for cows was defined as <0.4 mEq/L for prepartal period. However, there are no published data on the cutoff levels for NEFA in Turkish Saanen goats.

In the diagnosis of pregnancy toxemia in goats, BHBA threshold level should be accepted as 0.8 mmol/L in case the numbers of fetuses are not known. In case the numbers of fetuses are known, we need to consider the possible diseases in multiple pregnancies and BHBA measurements should be done and the threshold levels should be accepted as 1.1 mmol/L [43]. Bani Ismail et al.[44] stated that the BHBA level is accepted as 0.86-1.6 mmol/L for the subclinical toxemia of pregnancy in their study. However, there is no determined threshold level for BHBA for Turkish Saanen goats. We detected the minimum BHBA level on -30 day (0.62±0.04 mmol/L) and maximum on +15 day (0.85±0.05 mmol/L; P<0.05). BHBA level showed a linear increase from -30 day to +15 day (0.62±0.04 mmol/L; 0.70±0.04 mmol/L; 0.73±0.06 mmol/L; 0.85±0.05 mmol/L) and it decreased on the +30 day (0.70±0.06 mmol/L). Our findings are in line with the results of Sadjadian et al.[11]. These results indicate the gradual rise in the lipid mobilization till +15 day. Meanwhile, it was found that BHBA levels were higher than 0.8 mmol/L in 14 out of 60 animals (data were not presented) according to the cut-off BHBA levels, that is, there was a high hyper-ketonemia incidence.

In conclusion, this paper reports first results of changes in metabolic parameters and PON-1 levels during late pregnancy and early postpartum period in Turkish Saanen

goats. Conclusively, clear changes in levels of metabolic parameters and lipid mobilization were seen during the transition period. Significant changes in levels of PON-1 were occurred in Turkish Saanen goats and PON-1 may be used as oxidative stress biomarker. Further studies are required to determine the availability of PON-1 as a marker of oxidative stress and to confirm these findings. It should be taken into consideration that implementations of preventive strategies against oxidative stress and hyperketonemia could be useful to maintain health and production.

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Research Article

Characterization and Identification of Lactic Acid Bacteria by 16S rRNA Gene Sequence and Their Effect on the Fermentation Quality of Elephant Grass *(Pennisetum purpureum)* Silage

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Abstract

The purpose of this study is to isolate and identify lactic acid bacteria from elephant grass *(Pennisetum purpureum)* silage and examine their effect on the silage fermentation quality. Three of lactic acid bacteria strains were isolated from elephant silage and their characterization, identification, and influence on silage quality after 90 d of ensiling was studied. All three strains were Gram-positive, catalase-negative, and were grown in 6.5% NaCl and pH 4.00. Strains AZZ1, was identified as for genus *pediococcus*, whereas, AZZ4 and AZZ7 strains were classified as genus *Lactobacillus* according to the phenotype, 16S rRNA, and RecA gene analysis. Three strains were used as additives at 1.0×10^6 CFU/g of fresh material of elephant grass. Strain AZZ4 is found to be the most effective in improving the fermentation quality of the elephant grass silage, as indicated by a lower ($P<0.0001$) dry matter losses, pH value, water-soluble carbohydrates, acetic acid content, butyric acid, propionic acid and ammonia-N. However, the lactic acid content was higher ($P<0.0001$) compared to the control and other treatments. In conclusion, these results suggest that, for well-preserved silage, the isolates may be useful as inoculants for silage making, and could play a major role in developing silage production.

Keywords: *Isolation, Identification, Lactic Acid Bacteria, Sequence, Silage Quality*

16S rRNA Gen Sekansı Kullanılarak Laktik Asit Bakterilerinin Fil Çimeninde *(Pennisetum Purpureum)* Karakterizasyonu, Tanımlanması ve Fermantasyon Kalitesine Etkileri

Özet

Bu çalışmanın amacı Fil çimeninden *(Pennisetum purpureum)* laktik asit bakterilerin izolasyonu, tanımlanması ve silaj fermantasyon kalitesine etkilerini araştırmaktır. Üç laktik asit bakteri suşu fil silajından izole edildi ve bunların karakterizasyonu, tanımlanması ve 90 gün sonrasında silaj kalitesi üzerine etkileri çalışıldı. Üç suşun hepsi de Gram-pozitif ve katalaz-negatif olup %6.5 NaCl ve pH 4.00'de büyüme gösterdi. 16S rRNA ve RecA gen analiz sonuçlarına göre AZZ1 suşları pediococcus genusunda tanımlanırken AZZ4 ve AZZ7 suşları *Lactobacillus* genusunda sınıflandırıldı. Üç suş taze fil çimenine 1.0×10^6 CFU/g oranında katılarak kullanıldı. AZZ4 suşu daha düşük kuru madde kaybı ($P<0.0001$), pH değeri, suda çözünür karbohidratlar, asetik asit, burik asit, propiyonik asit ve amonyum-N miktarları baz alındığında fil çimen silajının fermantasyon kalitesini artırmada en etkili olan olarak belirlendi. Ancak laktik asit miktarı kontrol ve diğer uygulamalar ile karşılaştırıldığında daha yüksek olarak tespit edildi ($P<0.0001$). Sonuç olarak, iyi saklanmış bir silaj için elde edilen izolatların silaj yapımında faydalı olabileceği ve silaj üretimini geliştirmede önemli olabileceği düşünülmektedir.

Anahtar sözcükler: *İzolasyon, Tanımlama, Laktik asit bakterileri, Sekans, Silaj kalitesi*

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INTRODUCTION

Elephant grass *(Pennisetum purpureum)* is a monocot C4 perennial grass in the Poaceae family, and it is among the highest yielding tropical grasses. However, its high cell walls and low water-soluble carbohydrates can cause a significant inhibition of the ensiling process. The dry matter yields of elephant grass vary between 15 and 30 ton ha^{-1} a year [1]. Despite this, this grass concentrates too much moisture content at its best nutritional stage, which caused a reduction in the silage quality due to the high proteolysis; moreover, there are substantial nutrient losses due to such moisture levels [2]. Nevertheless, elephant grass is regarded as one of the most important tropical forages because of its high potential for biomass production, easy adaptation to diverse ecosystems and good acceptance by animals. Furthermore, it is extensively used to feed herds in the form of grazing [1]. However, its low crude protein content [3] and high structural carbohydrate contents [4] usually lead to the low nutritive value of silage. Recently, to avoid the aerobic deterioration of silage, heterofermentative LAB species, such as *L. buchneri* and, *L. brevis* have been developed as silage additives [5].

Lactic acid fermentation, an ancient conservation technique, currently preferred as a "natural" procedure to improve the products (dairy, vegetable, and meat) [6]. Epiphytic LAB colonies on plant material are responsible for the metabolism of water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid, thus resulting in pH decline and subsequent forage preservation [7]. There is a few number of homofermentative LAB commonly used in silage inoculants, including *Lactobacillus acidophilus, Lactobacillus plantarum, Enterococcus faecium and Pediococcus acidilactici* [8]. Commercially available microbial inoculant contains one or more of these bacteria, is selected based on their ability to achieve efficient fermentation. The silage quality could be improved by the addition of inoculants, then lactic acid production occurs more rapidly, thereby, the loss of nutrients during ensilage can be reduced [9]. Therefore, homofermentative bacteria can be used to improve silage preservation by accelerating and promoting the initial stage of the conservation procedure through the fermentation of water-soluble carbohydrates (WSC) into lactic acid followed by a rapid reduction in pH [10].

The processes of isolation, screening and identification of lactic acid bacteria from natural sources are routinely employed as the best means of gaining useful and genetically-stable strains for industrially-important products [11]. LAB that has industrial potential should be homofermentative, *i.e.*, they mainly produce lactic acid [11]. Facile recovery of lactic acid and consequent purification is also a main requirement in a homofermentative process [11].

To our knowledge, no information is available on the microbial ecology isolated from elephant silage, especially about the indigenous LAB and their effects during the fermentation process.

This study set out to isolate, screen and identify lactic acid bacteria colonizing elephant silage during the fermentation process. Isolated strains were identified biochemically, and selected strains were identified depending on the phenotype, 16S rRNA, and RecA gene analysis of sequence amplification product. As well as, some of the assessed to excellent LAB strains were used to inoculate elephant silage to determine their influence on the fermentation quality.

MATERIAL and METHODS

Isolation of LAB from Elephant Silage

Ten grams of elephant grass fresh material were blended with 90 mL of sterilized saline solution (8.50 g L^{-1} NaCl) and serially diluted from 10^{-1} to 10^{-6} in sterilized water. The number of the LAB were measured by the plate count method described by Cai et al.[12]. Three strains were isolated from elephant silage with GYP agar, and Lactobacilli MRS broth (Difco Laboratories, Detroit, MI) containing 1.5% agar incubated at 37°C for 2 d under anaerobic conditions. The separate colonies with different morphology were picked using a tooth pick and grown in MRS broth. Three strains were selected randomly from the plates containing between 30 and 300 colonies and purified by MRS agar, and each LAB strain was isolated and purified twice by streaking on MRS agar plates [12]. Pure cultures were grown on MRS agar at 37°C for 48 h, before being shifted to nutrient broth (Difco) and kept as standard cultures at -80°C with 10% glycerol for further examination.

Morphological and Physiological Tests of LAB

Morphological, physiological, gram-staining, catalase activity and gas production from glucose were determined according to the method described by Kozaki et al.[13]. Growth at different temperatures was observed in MRS broth after incubation at 5°C and 10°C for 14 days and at 45°C and 50°C for 7 days. Whereas, the growth at different pH 3.0, 3.5, 4.0, 4.5,7 and 8.0 was examined in MRS broth (the pH was adjusted with HCl or NaOH), after incubation at 30°C for 48 h. The acidity resistance of the LAB was tested in MRS broth containing 3.0 and 6.5% NaCl at 30°C for 2 days [10]. A preliminary identification assay of the LAB isolates based on the phenotypic characteristics was achieved according to the criteria of Bergey's Manual of Determinative Bacteriology. The purified strains were cultured on MRS plates in anaerobic vessels, and the obtained colonies were grown in 5 mL MRS media at 30°C overnight. A cell suspension was applied to API 50 CH strip wells (bioM_erieux. L'Etoile, France), which were coated with liquid paraffin, and the strips were incubated at 30°C. The results were assessed after 24 h and verified after 48 h. The fermentation of carbohydrates in the medium

was indicated by a yellow color, except for esculine (dark brown), and color reactions were scored by a chart provided by the manufacturer.

Genomic DNA Extraction

The selected LAB isolates were cultured for 24 h in MRS agar at 30°C and used for DNA extraction and purification. Seven aliquots of 10 mL from the homogenates used for microbiological analysis were centrifuged at 13.000×g for 5 min at 4°C. Pellets were washed twice with phosphate saline buffer (PBS) and incubated at 37°C for 1 h in 400 µL of lysis buffer (0.2 mg/L sucrose, 1.5 g/L Tris-HCl, 3 g/L NaCl and 3 g/L EDTA) containing lysozyme (10 g/L). Then 20 µL of SDS solution (100 g/L) and 2 µL of proteinase K solution (20 g/L) were added to each tube, mixed gently and incubated at 37°C for 1.5 h. After incubation, DNA was extracted using the phenol: chloroform method and precipitated with ethanol and sodium acetate. The final DNA sample was resuspended in 100 µL of Tris-EDTA (TE) buffer containing RNase (0.1 g/L), and the quality of the extracts was checked on agarose gels (0.1 g/L) and quantified by spectrophotometry.

The 16S rRNA gene sequence coding region was amplified by PCR thermocycler (Takara Shuzo Co., Ltd., Ohtsu, Japan) as described by Suzuki et al.[14]. PCR primers used were 27F (5′-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5′GGT TACCTTGTTACGACTT-3). The PCR was performed in a final volume of 50 µL using reagents provided by Bioline (London, UK). Initial DNA denaturation was performed at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 68°C for the 40s, and then a final elongation step at 68°C for 10 min. The expected size of the amplified fragments was verified on agarose gels (0.2 g/L).

The nucleotide sequences for the 16S rDNA described in this study were deposited in Gen Bank under accession no. of AZZ1, AZZ4, and AZZ7 were KY495875, KY584256, and KY584253, respectively.

Sequence Alignments and Phylogenetic Implications

The 16S rDNA sequences were aligned with 16S rRNA sequences from GenBank to identify organisms using nBLAST analysis. Then, the sequence information from representative organisms was introduced into the CLUSTALW program for assembly and alignment [15]. The 16S rRNA gene sequences of isolates were compared with sequences from LAB-type strains held in GenBank. Nucleotide substitution rates were calculated [16], and phylogenetic trees were constructed by the neighbor-joining method [17].

Silage Preparation and Treatment with LAB Strains

Elephant grass *(Pennisetum purpureum)* was harvested at dough stage from the experimental field of Nanjing Agricultural University Jiangsu, China (Latitude 32°01_19″ N, Longitude 118°51_08″ E, at Altitude 17 m above sea level). After harvesting, the grass was chopped manually to an approximate length of 2-3 cm. The grass was subsequently mixed homogeneously, packed, and compressed manually into approximately 1.32 L (9.5 cm diameter × 18.7 cm height), then ensiled in a laboratory silo and sealed airtight with a screw top. Strains AZZ1 *(Pediococcus acidilactici)*, AZZ4 *(Lactobacillus plantarum subsp. plantarum)*, AZZ7 *(Lactobacillus plantarum subsp. argentoratensis)* were selected as additives at 1.0×10^6 CFU/g of fresh material to elephant grass; then elephant grass were treated with (1) no additives (control), (2) AZZ1. (3) AZZ4 and AZZ7, control treatment was sprayed with equal distilled water. Additives were applied using a hand sprayer by spraying uniformly onto the mixture that was constantly hand mixed. After treating and thorough mixing, each treated batch was used to fill a silo, which was sealed with a screw top and plastic tape. A total of 12 laboratory silos were made (4 treatments × 3 replicates) for each treatment and kept at 25°C in ambient temperature. All silos were opened after 90 d of ensiling.

Chemical Analysis

On the sampling day, the content of each silo was removed, well-mixed, and divided into two silage samples. The first silage sample was used to determine the content of ammonia nitrogen (AN) and pH value, following the methodology used by Viana et al.[18], the other sample was dried using a forced-draft oven at 65°C for 48 h. For the chemical analysis of the fresh forage and silages, samples were finely ground to approximately 1 mm particle size in a Willey type laboratory mill in order to determine the dry matter (DM) content. The total nitrogen (TN) content of the fresh forage and silage samples was examined according to the Kjeldahl method [19]. Crude protein (CP) was analyzed and calculated as the TN multiplied by 6.25. Water-soluble carbohydrates (WSC) contents were determined by a colorimetric method after reacting with an anthrone reagent [20]. The neutral detergent fiber and acid detergent fiber content were performed according to the techniques described by Van Soest et al.[21]. Organic acids contents of silage, including the lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid (BA) were analyzed by high-performance liquid chromatography (Agilent Technologies, CA, USA) according to the methods described by Liu et al.[22]. Ammonia nitrogen (AN) was measured according to the method of phenol-hypochlorite reaction [20]. The pH of fresh forage and silage were measured using a pH meter (F-23; Horiba, Tokyo, Japan).

Statistical Analyses

Silage fermentation data were analyzed as a completely randomized design using the general linear model (GLM) procedure of Statistical Analysis System [23]. Least significant

difference was used to separate means when significant effects (P<0.05) were detected.

RESULTS

The Morphological and Physiological Properties of Characteristic Strains Isolated from Elephant Silage

All isolates were identified as gram-positive, catalase negative, rod-shaped bacteria, and their morphological and physiological characteristics are presented in *Table 1*. Strains AZZ1 and AZZ4, grew normally at 10°C, whereas, AZZ7 grew weakly at 10°C and strains AZZ1 grew weakly at 15°C, strain AZZ1 was unable to grew at 45°C while AZZ4 and AZZ7 were grow. Moreover, no growth was detected at 50°C. All strains were able to grew at pH 3.5. Apart from the weak growth of AZZ4 and AZZ7 at pH 3.5, all strains could grow in the rang of pH 3.5-8.0, however, the growth of all strains seems to be inhibited at pH 3.0. All strains showed homofermentative products, and all were able to fermented glucose and fructose and other sugars.

16S rRNA Gene Sequence Analysis

After blasting the 16S rRNA sequence, Strains AZZ4 and AZZ7 were clustered in the genus *Lactobacillus* with 99% similarity among their 16S rDNA gene sequences, on the other hand, strain AZZ1 was clustered in the genus *pediococcus* with 98% similarity in their 16S rDNA gene sequences, as shown in the phylogenetic tree *(Fig. 1*, bootstrap between 51%-100%).

Amplification Products Obtained from the recA Gene Multiplex Assay

Fig. 2 shows the amplification products obtained from the recA multiplex assay; lane M contained a 600 bp PLUS DNA ladder (Tiangen Biotech Co, Ltd., Beijing, China). Lanes 1, 2, and 3, are PCR amplification products from *Pediococcus acidilactici*, (AZZ1), *L. plantarum* subsp. *Plantarum* (AZZ4), *L. plantarum subsp. Argentoratensis* (AZZ7), respectively. Sequences of the three strains were deposited with GenBank under accession numbers of KY495875, KY584256 and KY584253 for AZZ1, AZZ4 and AZZ7, respectively.

Characteristics of Elephant Grass Before Ensiling

The chemical and microbiological compositions of fresh elephant grass before ensiling are shown in *Table 2*. The number of lactic acid bacteria was low on the material before ensiling which is common for tropical grasses to have a low number of lactic bacteria, less than 10^6 CFU/g fresh forage

Effects of LAB Isolate on Silage Quality After 90 d of Ensiling

The effects of LAB strains on the fermentation quality of elephant silage after 90 d of ensiling are shown in *Table 3*. At the ensiling day, the analysis of the chemical composition

Table 1. *The characteristics and sugar fermentation patterns of lactic acid bacteria isolates used for silage inoculation*

Characteristic	AZZ1	AZZ4	AZZ7
Shape	Rod	Rod	Rod
Gram stain	+	+	+
Gas from glucose	-	-	-
Fermentation type	Homo	Homo	Homo
Catalase activity	-	-	-
Growth at 5°C	-	-	-
Growth at 10°C	+	+	W
Growth at 15°C	W	+	-
Growth at 45°C	-	+	+
Growth at 50°C	-	-	-
Growth in 3.5%NaCl	+	+	+
Growth in 6.5%NaCl	+	+	+
Growth at pH 3.0	-	-	-
Growth at pH 3.5	+	+	w
Growth at pH 4.0	+	+	+
Growth at pH 4.5	+	+	+
Growth at pH 7.0	+	+	+
Growth at pH 8.0	+	+	+
Carbohydrate fermentation			
L-Arabinose	+	+	+
Ribose	+	+	+
D-Xylose	+	+	+
ß-Methyl-xyloside	-	+	+
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	+	+	+
Rhamnose	+	-	-
Inositol	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
α-Methyl-D-mannoside	-	+	+
α-Methyl-D-glucoside	-	+	+
N-Acetyl glucosamine	+	+	+
Amygdalin	+	+	+
Esculin	+	+	+
Salicine	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Saccharose	+	+	+
Melezitose	+	+	+
D-Raffinose	-	+	-
ß-Gentiobiose	+	+	+
D-Tagatose	+	-	+
Gluconate	+	+	+

+, normal growth; -, no growth; ***w,*** *weak growth;* ***Homo,*** *homofermentative;* ***Hetero,*** *heterofermentative*

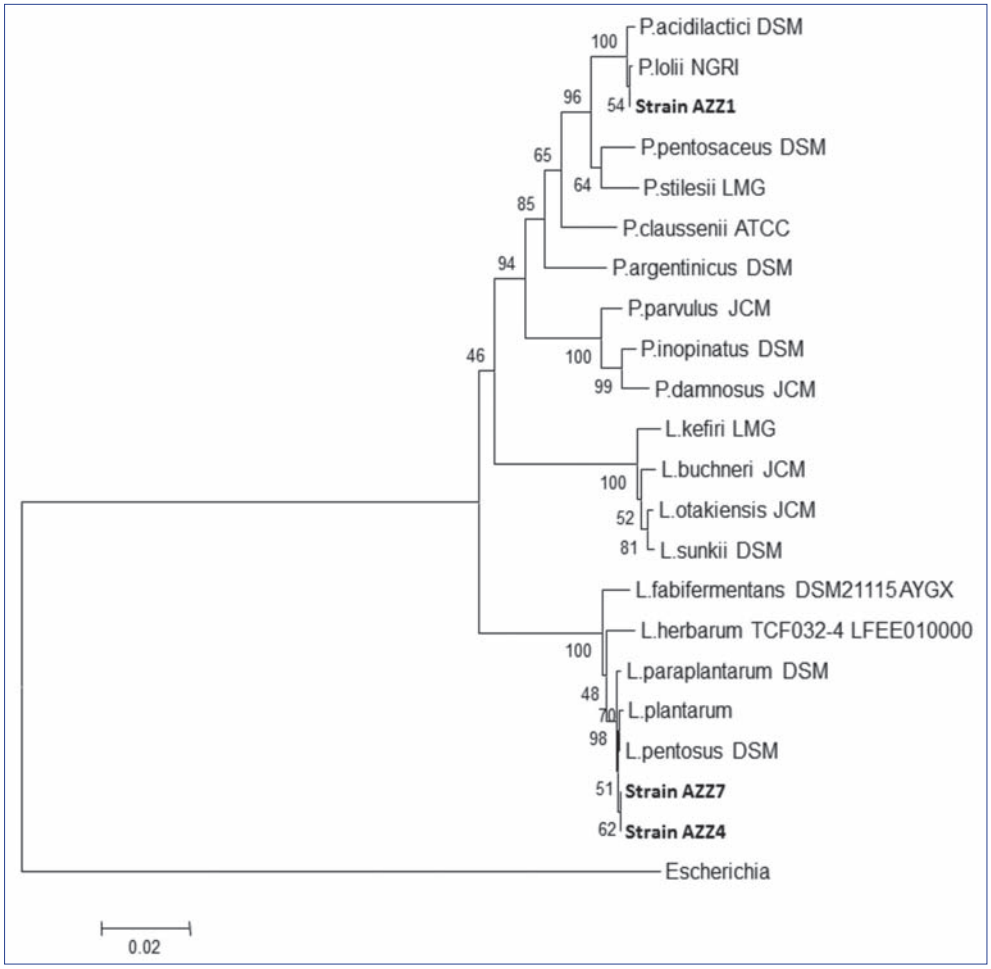

Fig 1. Polygenetic tree showing the relative positions of strains AZZ1, AZZ4, and AZZ7 isolate from elephant silage and related species, as inferred by the neighbor-joining method using complete 16S rRNA sequences. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. *B. subtilis* is used as an outgroup. The GenBank accession no of the 16S rRNA gene of each strain is shown behind the strain

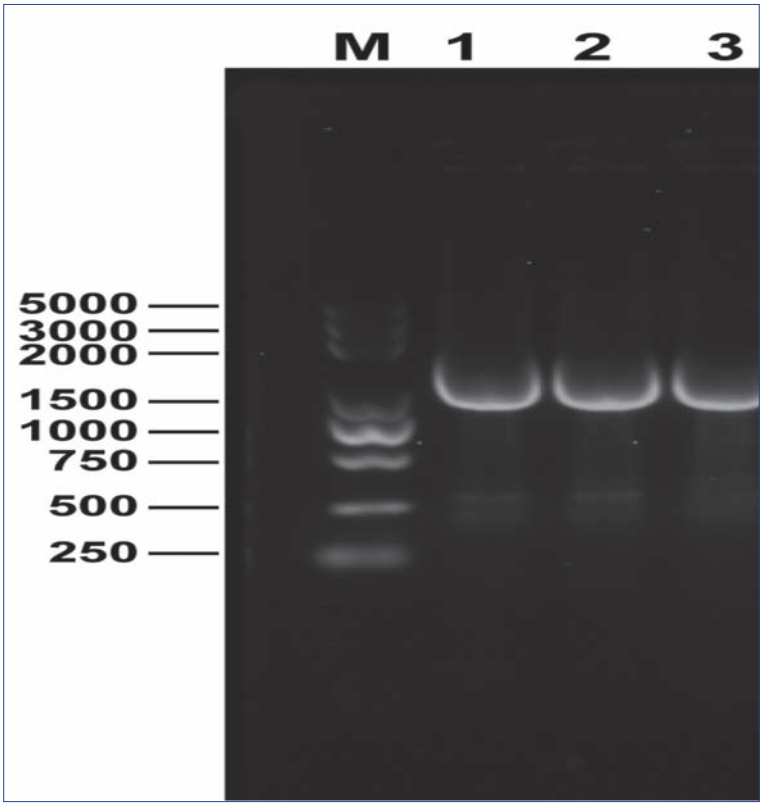

Fig 2. Amplification products obtained from the recA multiplex assay. Lane M contained a 600 bp PLUS DNA ladder (Tiangen Biotech Co, Ltd., Beijing, China). Lanes 1, 2 and 3, PCR amplification products from *Pediococcus acidilactici*, (AZZ1), L. *plantarum* subsp. *Plantarum* (AZZ4), *L. plantarum subsp. Argentoratensis (AZZ7)*, respectively

Table 2. *Chemical and microbial composition of elephant grass before ensiling*

Component	Content
Dry matter (g/kg FM)	286.5
pH	5.97
Crude protein (g-kg DM)	272.6
Neutral detergent fiber (g/kg DM)	697.4
Acid detergent fibre (g/kg DM)	390.3
Water-soluble carbohydrates (g/kg DM)	55.6
Buffering capacity (mEq/kg DM)	284
Lactic acid bacteria (log^{10} CFU/g FM)	4.74
Aerobic bacteria (log10 CFU/g FM)	6.53
Mould (log^{10} CFU/g FM)	4.36
Yeast (log^{10} CFU/g FM)	4. 85

FM, *fresh matter;* ***DM,*** *dry matter;* ***mEq,*** *milligram equivalent;* ***CFU,*** *colony-forming units*

phenotypic methods is inconsistently successful [24], while the reported use of 16S rRNA sequence analysis is considered a good approach to identify LAB strains at both the genus and species level. However, some LAB species, for example, *L. plantarum* and *L. pentosus* have very similar 16S rRNA gene sequences, differing only by 2 bp [25]. This finding is in agreement with Pang et al.[26] who found carbohydrate fermentation patterns showed ambiguity. Although the pattern of strains isolated from silage and two type strains *L. pentosus* and *L. plantarum* were quite similar, they could not be identified at the species level based on the 16S rRNA gene sequence and API 50 CHL analysis. Therefore, other phylogenetic analysis methods were required to distinguish these strains accurately. In the present study, the selected strains were gram-positive, catalase-negative rods that produced major metabolic products such as lactate from glucose.

Following phylogenetic analysis of 16S rRNA gene

Table 3. *Inoculating effects of LAB strains on the fermentation quality of elephant grass silage at 90 d of ensiling*

Item	Treatment				SEM	P-value
	Control	AZZ1	AZZ4	AZZ7		
DM (g/kgDM)	244.03^{a}	235.35^{c}	239.31^{b}	236.34^{bc}	0.290	0.0004
pH	5.42^{a}	3.19^{b}	3.12^{b}	3.46^{b}	1.080	<.0001
Lactic acid (g/kg DM)	30.26^{d}	47.74^{c}	61.70^{a}	57.82^{b}	3.680	<.0001
Acetic acid (g/kg DM)	19.43^{a}	15.26^{b}	8.31^{d}	11.51^{c}	1.286	<.0001
Propionic acid (g/kg DM	5.19^{a}	2.44^{b}	1.41^{c}	1.48^{c}	0.467	<.0001
Butyric acid (g/kg DM)	8.98^{a}	4.33^{b}	2.08^{b}	3.39^{b}	0.812	<.0001
Water soluble carbohydrates (g/kgDM)	5.63^{a}	3.97^{c}	4.93^{b}	4.75^{b}	0.183	<.0001
Ammonia nitrogen (g/kg total nitrogen)	55.36^{a}	37.66^{b}	26.90^{c}	35.46^{b}	3.217	<.0001
LAB (log cfu/g of FM)	4.78^{b}	5.82^{a}	5.90^{a}	5.70^{a}	0.145	0.0008
Aerobic bacteria (log cfu/g of FM)	6.65a	6.48a	5.58^{b}	6.21^{a}	0.134	0.0024
Yeasts (log cfu/g of FM)	5.50^{a}	5.23^{ab}	4.91^{b}	5.13^{ab}	0.074	0.0116

DM, *dry matter;* ***FM,*** *fresh matter;* ***log,*** *denary logarithm of the numbers;* ***cfu,*** *colony-forming units;* ***LAB,*** *lactic acid bacteria;* ^{a,b,c,d} *Significant differences in means when used multiple comparison* based on *Tukey's test (P<0.05) within the control silages over the 90 day fermentation; Control;* ***AZZ1,*** *Pediococcus acidilactici;* ***AZZ4,*** *Lactobacillus plantarum subsp. plantarum;* ***AZZ7*** *Lactobacillus plantarum subsp. argentoratensis;* ***SEM,*** *standard error of means*

revealed that the contents of DM in the AZZ4 treated silages presented a significant difference (P=0.0004) compared with other treatments. The comparison of the fermentation quality compared with the control showed that all LAB treated silages presented lower values of pH, Water soluble carbohydrates, butyric acid, propionic acid, and NH_3-N content (P<0.0001), while higher lactic acid content and LAB count (P<0.0001) were revealed. Additionally, the AZZ4 treated silages exhibited the lowest values of pH, propionic acid, butyric acids and NH_3-N content compared with all other treatments and control samples.

DISCUSSION

Differentiation between isolates of known species using sequences, selected strains AZZ4, and AZZ7 were identified as for genus *Lactobacillus* and AZZ1 was identified as the genus *Pediococcus*. However, they could not be identified to the species level by phenotypic characteristics. There have been several reports of *Lactobacilli* composing the dominant microbial population of forage crops and silage, where they may contribute to silage fermentation. Some silage-associated lactobacilli have been characterized by phenotypic features and 16S rRNA gene sequences and have been described as different species: for example, *L. paraplantarum, L. brevis, L. buchneri, L. acidophilus, L. plantarum, L. fermentum, L. casei and L. pentosus* [8,10,12,26,27].

In recent years, the phylogenetic relationships of the LAB have been studied extensively in 16S rDNA sequence

ribotyping, and DNA-DNA hybridization experiments and a new species *L. nasuensis* isolated from silage has been added [28]. In the present study, the strains AZZ1, AZZ4 and AZZ7 had a high similarity (>98%) of 16S rDNA sequences to their corresponding known strains within the *Lactobacillus* and *Pediococcus* families, and furthermore confirming that they are most closely related to *L. plantarum* and *P. acidilactici* genera.

The lactic acid bacteria species identified in this study were natural populations of a diversity of forage crops and silages. This finding was in agreement with the results of other studies, which demonstrated that the natural fermentation methods in grass silages and forage crop are dominated by *Enterococcus, Lactococcus, Leuconostoc, Pediococcus and Lactobacillus* species [6,29]. Additionally, a study of Ennahar et al.[8] reported that the LAB species of paddy rice silage in Japan also included *Pediococcus*, which is in consistence with the current study findings. The main reason might be that, the bacterial colonization of plants and fresh crops is controlled by many factors, most notably the climate variations [30]. Therefore, examining the microflora of elephant grass samples from various locations could provide more information about the LAB inhabitation and facilitate the optimization of inoculant characteristics to in order to achieve better silage fermentation quality.

The aim of adding LAB inoculants at ensiling is to guarantee rapid and vigorous fermentation, which leads to faster accumulation of lactic acid, lower pH values during the early stages of ensiling, and inhibition of some pathogenic bacterial growth [31]. On the first stage of the screening method (data not shown), three strains (AZZ1, AZZ4, and AZZ7) produced the highest amounts of lactic acid in the elephant broth and were subsequently selected as the inoculants for elephant silage. All selected isolates increased the lactic acid content and the number of lactic acid bacteria, while decreased, the pH, ammonia nitrogen, water-soluble carbohydrates, butyric acid, and propionic acid when compared to the control group. A small amount of butyric acid was detected except in the control and fresh treatments. A decreased of butyric acid could be related to the reduction in pH caused by the addition of the isolates, which may have inhibited the growth and proteolytic activity of microorganisms such as Clostridia [32,33]. It is accepted that proteolytic activity decreases in the ensiled forage at lower pH.

The low pH values could also explain the decrease in NH_3-N content in response to treatment with the isolated strains. Similar results were reported previously [34,35]. According to the outcomes of Cai et al.[31], *L. plantarum* FG10, a LAB strain isolated from Italian ryegrass, increased the lactic acid content and decreased the butyric acid, NH_3-N content and DM loss of Italian ryegrass silage [10]. This improvement of silage quality was observed when AZZ1, AZZ4, and AZZ7 strains where employed for silage fermentation. Lactic acid is the main reason for the pH decline in high silage quality.

In general, a typical selection criteria for the LAB in silage fermentation would be the ability to survive in various fermentation environments, the ability to produce lactic acid as the main product of carbohydrate fermentation, as well as the competitiveness between inoculants and natural population. Our study evaluated the effects of various lactic acid bacteria on silage quality and found that, the inoculant effects varies at the strain level within species [36]. Also, some studies have found a significant correlation between silage inoculated with LAB and animal performance. Cao et al.[37] found that, *L. plantarum* inoculated with vegetable residue silage had the lowest methane production and highest in vitro DM digestibility [37].

In conclusion; this study showed the isolation of naturally evolved LAB species in the elephant silage and the positive effects of inoculating the silage regarding quality and preservation, which leads to a good feed source for livestock diets. *Lactobacillus Plantarum* subsp. *plantarum* (AZZ4) presented the highest content of lactic acid and lowest NH4-N and pH value among the three inoculated LAB stains, thereby, it could be regarded as a suitable strain for improving elephant silage quality and, thus, is a potential inoculant for the production of silage.

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Conflict of Interest

All author declares that they are no conflict of interest.

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Research Article

Using of Pelleted and Extruded Foods in Dog Feeding [1] [2]

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Abstract

This study was carried out to determine the effects of pellet and extruded foods on gelatinization, digestibility and faecal quality of dogs. In study 30 adult male dogs of mixed breed, weighing 15-30 kg, neutered and around 1-3 of age were used. The two tested dog-food formulations had the same composition, but one was produced in pellet form, while the other was extruded in a private factory. Feeding experiments were conducted at the Dog Unit of the Veterinary Faculty in Selçuk University. Pelleted and extruded food contained 4.87% and 17.81% gelatinized starch, respectively (P<0.001). Tested dogs preferred extruded food at a rate of 0.66. The digestibility, faecal score, and cost of pelleted, extruded, imported, and domestic dog food were compared. The most common commercial brands were selected for the latter two categories. Based on faecal samples, the dry matter digestibility of the four dog-food types was 81.2%, 84.2%, 83.7%, 83.5% (P<0.05) respectively. The faecal score was 3.48 for dogs that consumed pelleted food and 3.68-3.91 for dogs fed the other three extruded foods. Cost calculations revealed that extruded food is five times more economical than imported food.

Keywords: *Extruded, Dog food, Digestibility, Pellet, Preference*

Pelet ve Ekstrude Mamaların Köpek Beslemede Kullanılması

Özet

Bu çalışma pelet ve ekstrude formda üretilen mamaların jelatinizasyon, köpeklerde sindirilebilirlik ve dışkı kalitesine etkilerinin belirlenmesi amacıyla yapıldı. Çalışmada 15-30 kg ağırlıkta kısırlaştırılmış 30 adet 1-3 yaşlı karışık ırk erkek köpek kullanıldı. Bileşimi aynı olan iki formülün biri pelet şeklinde, diğeri ekstrude formda özel bir tesiste üretildi. Yedirme denemeleri Selçuk Üniversitesi Veteriner Fakültesi Köpekçilik Ünitesinde yürütüldü. Jelatinize nişasta oranı pelet mamada %4.87, ekstrude mamada %17.81 (P<0.001) bulundu. Ekstrude mamanın köpekler tarafından tercih edilme oranı 0.66 olarak tespit edildi. Bu çalışmada üretilen pelet ve ekstrude mamalar, piyasada en çok bilinen biri ithal ve biri yerli olan ticari mamalar ile sindirilebilirlik, dışkı skoru ve maliyet bakımından karşılaştırıldı. Pelet, ekstrude, ithal ve yerli ticari mamaların dışkı toplama yöntemiyle belirlenen kuru madde sindirilebilirlikleri sırasıyla %81.2, 84.2, 83.7, 83.5 (P<0.05) olarak tespit edildi. Dışkı skoru pelet mama tüketenlerde 3.48 bulundu, diğer üç ekstrude mamaları tüketenlerde 3.68-3.91 arasında idi. Maliyet hesaplamasında bu çalışmada üretilen ekstrude mama ile köpek beslemenin ithal mamadan 5 kat daha ekonomik olabileceği belirlendi.

Anahtar sözcükler: *Ekstrude, Köpek maması, Pelet, Sindirilebilirlik, Tercih*

INTRODUCTION

The dog food industry of Turkey has experienced rapid progress in recent years, with a rising number of entrepreneurs interested in producing dog food. At present, dog nutritional needs are largely met with imported food, which can be purchased off the internet and vary greatly in price. Due to inconsistent prices and a desire for natural products, dog owners tend to mix home-grown dog food as an inexpensive, domestic, and higher quality alternative.

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Whether homemade or commercial, dog food formulas contain a considerable amount of cereal, with rice and corn being the most common. Due to high starch content, grains are mainly used as an economical energy source, while also acting as a swelling and bonding agent. However, raw starch has very low digestibility and must be gelatinized or cooked during food production. Homemade recipes tend to boil grains, whereas commercial foods process grains in an extruder.

Gelatinization is a mechanical process that deteriorates starch crystal structure through altering moisture, temperature, and pressure conditions, causing starch granules to swell [1]. Starch gelatinization and the form of the final product are both taken into account when measuring dry food quality [2].

Extrusion has been used in the food industry and animal-feed production since the 1950s. The aqueous food mixture undergoes heat treatment and is forced through the die with a spiral screw, resulting in a product of a particular shape, such as strips that are then cut and then dried. To enhance flavour, the product is typically sprayed with oil or similar compounds. During extrusion, products experience up to 200°C in as little as 270 s, causing major chemical and physical changes that result in swelling (similar to making popcorn). For optimal protein quality in dog food production, extrusion conditions were found to be 110-150°C, with a 300 g/kg moisture content, and 120-160°C drying temperature [3,4]. During this process, undesired enzymes are denatured, anti-nutritional factors are destroyed, and food is sterilised, all without impairing the natural odour and taste [5-8]. Extrusion also significantly increases digestible starch [9], but may also cause undesirable effects, such as vitamin (A, E, thiamine) deficiencies, lipid oxidation, and a reduction in amino acid content through the Maillard reaction [6].

An important method for assessing dog health involves examining stool amount and consistency. High digestibility is a desirable quality for dog food [10], and stool characteristics act as a useful indicator of digestion levels.

In addition to digestibility, dog foods must also be palatable. Various methods are available for determining dog preferences, with the two-pan palatability test being widely used. This test involves presenting two foods simultaneously before the animal and observing consumption. After a set duration, the remaining food is measured and the preference rate is calculated [11,12].

This study had three objectives. First, we determined the suitability of pelleted dog foods commonly used in temporary care and rehabilitation centres. Second, we aimed to demonstrate that domestic extruded foods can be produced commercially. Finally, we evaluated the quality of several manufactured foods via feeding experiments in dogs.

MATERIAL and METHODS

Animals and Management

Experimental procedures were approved by the local ethics committee (No: 2014/53) at the Dog Unit of Selçuk University's Veterinary Faculty. Dogs were housed in individual cells with concrete floors, each containing a 190 × 190-cm enclosure and a 510 × 230-cm open area.

Subjects were 30 neutered, adult male dogs, of mixed breed and around 1-3 of age. Exposure of the dogs to general health conditions and internal and external parasitic practices were routinely performed. Dogs weighed around 15-30 kg.

Dogs were fed daily at the same time. Food and water were provided in 90-oz metal pans and ceramic bowls, respectively. The containers and housing were washed weekly with pressurised water.

Dog Food Production

Tested dog food formulations contained the same raw material and nutrient composition *(Table 1)*. One was pelleted and the other was processed with an extruder. Both were produced at Bil-Yem Facilities.

Food ingredients were first weighed and then milled to pass through a 0.4 mm sieve. Water was added to

Table 1. *Composition of pellet and extrude dog foods*

Ingredients	**%**
Poultry meal	15.00
Barley	10.00
Corn	27.00
Corn gluten meal	13.00
Corn starch	10.78
Rice	15.00
Whey	2.00
Sunflower oil	3.00
Beef tallow	3.00
Vitamin-mineral*	1.22
Calculated nutrients/100 g DM	
Crude protein, g	23.50
Energy, kcal	445
Crude fibre, g	2.27
Ash, g	4.25
Carbohydrate, g	62.53
Calcium, g	0.64
Phosphorus, g	0.61
Fat, g	10.80
Linoleic acid, g	2.02

** Aminovit, minesol, K chloride, Zn proteinate, Ca iodate, Na bicarbonate*

the ingredients to maintain moisture at around 25%, processed in a mixer for 20-30 min, and then added to a double-screw extruder. Extruder internal temperature was raised from 90°C to 135°C in four steps; the contents were cooked for a maximum of 4 min. Dog food was then moved to a conveyer-belt dryer and subjected to temperatures peaking at 148°C for 30-45 min. The resulting product was sprayed with oil, vitamins, and mineral additives in the lubricating unit before being cooled, then stored in 15-kg bags. The same formulation was passed through the pelletizing unit (70-80°C, 18% moisture) to obtain a 6 mm pellets.

Nutrient Analyses

Dog foods were ground in a Retsch SM100 laboratory mill and passed through 0.5 mm sieves. Analyses of dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fibre (CF), and starch were performed following AOAC methods [13]. The resultant data were used to calculate the metabolic energy in both products [14]:

$$\text{ME-NRC, kcal/kg} = ((5.7 \times CP \times 10) + (9.4 \times EE \times 10) + (4.1 \times (NFE \times 10 + CF \times 10))) \times (91.2 - (1.43 \times CF))/100 - (1.04 \times CP \times 10),$$

where CP is crude protein, EE is ether extract, NFE is nitrogen-free extract, and CF is crude fibre.

Gelatinized Starch Ratio

To determine the effect of pelleting and extrusion on starch degradation, three repetitive gelatinized starch measurements were made with a spectrophotometer, using a starch damage assay kit (Megazyme International Ireland 2014). This procedure has been adopted by the American Association of Cereal Chemists (AACC Method 76-31.01) and the International Association for Cereal Science and Technology (ICC Method No. 164). In the procedure, damaged starch granules are hydrated and hydrolysed to maltosaccharides plus α-limit dextrins by controlled treatment with purified fungal α-amylase. The fungal α-amylase treatment is designed to give near complete solubilisation of damaged granules with minimum breakdown of undamaged granules. This reaction is terminated on addition of dilute sulphuric acid, and aliquots are treated with excess levels of purified amyloglucosidase to give complete degradation of starch-derived dextrins to glucose. The glucose is specifically measured with a high purity glucose oxidase/peroxidase reagent mixture. Determined values are presented as starch (damaged) as a percentage of flour weight on an "as is" basis (Megazyme International Ireland 2014).

Preference Test

To determine whether dogs preferred pelleted or extruded foods, dogs were fed 500 g of each in two identical feeding pans, once per day (at the same time). The food pans were positioned at the same distance away from the subjects, so that they could reach either equally easily. Dogs were kept in the outer area of their compartments while the pans were being placed in the enclosure.

The dogs were given clean water ad libitum during the experiment. At the end of 1 h, both food pans were removed and weighed to determine how much was consumed. Potential directional preferences were eliminated by switching the pans' left/right positions daily until the end of the test (4 days). Food preference was calculated with the following equations [12,15]:

$$\text{Extruded food preference ratio, \%} = A/(A + B),$$

$$\text{Pellet food preference ratio, \%} = B/(A + B),$$

where A is the amount of consumed extruded food (g) and B is the amount of consumed pellet food (g).

Determination of Digestibility

The total collection method [10,16] was used to determine the digestibility of organic matter (OM), CP, EE, and CF in two commercial diets (one imported, one domestic). Four groups (seven dogs each) were separated using sensitive sorting [17], based on weight, body condition scores, and their location in the Dog Research Unit. Each group was fed a different type of food for 14 days. Subjects were given the same amount of food daily, around 3-8% of their maintenance requirements (according to their consumption levels during a nine-day acclimatisation period). Any remaining food was collected and weighed on the next day. Water was provided ad libitum. Twice a day during the last five days, faeces were collected with plastic scrapers, placed in nylon bags, weighed, and stored at -20°C. At the end of the trial, stool samples were dissolved, homogenised, and then weighed in aluminium containers to determine dry matter content. Next, samples were oven-dried (70°C) for 60 h, then ground for the analysis of ash, crude protein, ether extract, and crude fibre content. Nutrient digestibility was calculated using the following formulas:

$$\text{Dry matter digestibility, \%} = (\text{dry matter of food} - \text{dry matter of faeces})/\text{dry matter of food} \times 100,$$

$$\text{Nutrient digestibility, \%} = (\text{nutrient in food} - \text{nutrient in faeces})/\text{nutrient in food} \times 100.$$

Faecal Consistency

During the last 4 days of the digestibility trial, faecal consistency was scored as follows: 1. soft and unshaped stool; 2. soft and vaguely shaped stool; 3. soft, moist, and spotted stool with definite shape; 4. well-formed, undistorted, and non-marking stool; 5. well-formed, solid, and dry stool [18]. Scoring was conducted by three independent observers and a final average was taken.

Determination of Cost

Potential retail sale prices of the pelleted and extruded foods if offered as a commercial product were calculated to estimate costs. Ingredients prices and their value-added

taxes were determined using the diet formulation. Potential operating expenses, packing costs, production wastages, post-shipment waste, depreciation, and profitability expenses were added to the price. Calculated prices were then compared to the retail prices of commercial imported and domestic foods.

Statistical Analyses

Differences in nutrient digestibility and faecal scores across the dog food types were examined using ANOVA. Differences in the ratio of damaged starch were analysed with the Student's t-test. Means were separated with Duncan's multiple range tests. Significance was set at P<0.05. All analyses were performed in IBM SPSS Statistics (version 22, IBM Corp., Chicago, IL).

RESULTS

Table 2 shows the results of nutrient analysis, and *Table 3* shows the damaged starch ratios. Extruded food contained four times more gelatinized starch than pelleted food.

Table 4 shows the nutrient digestibility of four tested foods. Pelleted food contained the lowest DM and CP digestibility. Extruded food and the two commercial foods did not differ in their DM, OM, and CF digestibility. *Table 5* shows subject preferences for pelleted versus extruded food. On average, subject dogs consumed 199.85 g of pelleted and 380.33 g of extruded food daily, preferring extruded food by 66% and pelleted food by 34% (P<0.001). Finally, *Table 6* shows the faecal scores. The lowest faecal score (3.48) was obtained from animals consuming pellet food.

The retail price per kg of pelleted and extruded food was determined. *Table 7* compares the costs of the daily amount necessary to meet a 25-kg dog's energy requirements across the four tested foods. Because the foods differ in nutrient content, we calculated how much it would cost to meet the daily energy requirement of an adult dog of average weight. We found that the daily feeding cost was 1.13 TRY, 0.87 TRY, 5.78 TRY, and 1.33 TRY for extruded, pelleted, commercial imported, and commercial domestic food, respectively.

DISCUSSION

Although the pelleted and extruded foods were produced with the same formulation, the latter contained slightly less ash, CF, and EE than the former. The low ash and EE content may be due to a lack of precision in adjusting the amount of oil used when applying minerals during preparation. Regardless of the lower EE, dogs preferred extruded food over pelleted food. Furthermore, we observed that measured EE, CP, and energy levels were lower than the expected values based on the ingredients used in the formula, an outcome that could be attributed supplier overestimation of protein and fat content in the poultry meal and corn gluten meal used.

The gelatinized starch ratio in dog food varies between by 10-35% [19]. This study found a gelatinized starch content

Table 2. *Analysis results of foods, DM%*

Food	DM	Ash	EE	CF	CP	Starch	ME, kcal
Pelleted	92.81	4.88	9.23	2.66	22.76	51.72	393
Extruded	92.57	4.29	8.66	2.25	22.98	50.84	395
Commercial, imported	94.02	4.92	12.59	3.12	22.26	45.14	405
Commercial, native	94.34	5.97	9.39	3.12	26.52	42.28	388

DM: *Dry Matter*

Table 3. *Gelatinized starch in foods, %*

Food	X	Sx
Pellet	4.87	0.04
Extrude	17.81	0.18
P	<0.001	

Table 4. *Nutrient digestibility of foods, % (n=7)*

Food	DM	OM	EE	CF	CP
Pelleted	81.17±1.12^{b}	84.13±1.11^{b}	94.98±0.30^{ab}	26.77±3.60	76.05±1.60^{c}
Extruded	84.20±0.45^{a}	87.87±0.36^{a}	95.72±0.40^{a}	19.96±5.12	79.63±1.12^{b}
Commercial, imported	83.66±0.66^{a}	85.96±0.63^{ab}	95.29±0.26^{ab}	29.23±2.61	79.94±0.97^{b}
Commercial, native	83.47±0.52^{a}	87.7±0.39^{a}	94.16±0.38^{b}	19.79±2.90	85.62±0.59^{a}
P	0.039	0.003	0.029	0.235	<0.001

^{a,b} *Means within a row with no common letter differ significantly (P<0.05);* ***DM:*** *Dry Matter,* ***OM:*** *Organic Matter* ***EE:*** *Ether Extract,* ***CF:*** *Crude Fibre,* ***CP:*** *Crude Protein*

Table 5. Results of two-pan preference test (n=30)

Preference Test	Pellet	Extrude
Daily consumption, g	199.85	380.33
Preference rate, %	34	66

Table 6. Faecal scores of foods (n=7)

Food	Faecal Score	
	X	Sx
Pelleted	3.48	0.15
Extruded	3.68	0.21
Commercial, imported	3.91	0.15
Commercial, native	3.78	0.15
P	0.349	

Table 7. Daily food costs for a 25 kg adult dog

Food	Daily Intake, kg	Cost, TRY	Rate, %
Pelleted	0.368	0.87	77
Extruded	0.366	1.13	100
Commercial, imported	0.351	5.78	510
Commercial, native	0.383	1.33	118

of 17.81% and 4.87% in extruded and pelleted food, respectively. The degree of gelatinization in extruded food reached the desired limit, indicating greater starch digestibility. Digestion trial results corroborate this conclusion. In sum, an increase in starch gelatinization shows that the dry and organic matter in extruded food has higher digestibility. Furthermore, pelleted food was less digestible because it was not sufficiently gelatinized.

We expected that the high-heat treatment would increase gelatinized starch, and therefore the digestibility of dry and organic matter. In a comparison with commercial foods, dry matter digestibility was highest in extruded food and lowest in pelleted food, with commercial foods in between. The higher dry matter digestibility was likely due to the fact that commercial foods are also extruded and have higher starch digestibility. Overall, obtained digestibility values are within previously reported limits [20-25].

Organic matter digestibility was higher in extruded food than pelleted food. Also, it was higher in commercial domestic food than pellet food. These are generally in line with previous work using poultry by-product meal and poultry meal as the source of animal protein in dog foods, resulting in an OM digestibility of 87.0-88.8% [20]. In other studies [20,22,23,25], OM digestibility ranged widely between 86.3% and 92.8%. We also found that EE digestibility ranged from 94.16% to 95.72%, with the lowest values in commercial foods. This outcome is probably due to the use of animal fat, which is difficult to digest. Generally, the fat digestibility of dog food varies between 91.7% and 95.5% [20,22,23,25].

The ability of dogs to digest crude fibre is fairly low. Indeed, CF digestibility was reported to be 38.3% on average (across 259 dog foods) in one study [26]. Greater CF digestibility increases stool consistency in dogs. In this study, CF digestibility was varied between 19.79% and 29.23%.

Observed protein digestibility was between 76.05% and 85.62%. The protein digestibility were significantly greater in commercial domestic food than in the other three foods, with pelleted food exhibiting the lowest values. It is known that the processing of extrusion increases protein digestibility [3,6]. In this study, CP digestibility of 76.05% in pellet food increased to 79.63% in extruded food with the same formula, which means an increase of 4.71%. The level of protein in commercial domestic extruded food is higher than other foods. However, a high protein level in the food does not necessarily mean increased digestibility, as proteins with low digestibility (e.g. vegetable sources) can also be elevated. A previous study found that the ability of dogs to digest protein did not differ when fed with foods containing 25% and 35% (dry matter basis) crude protein [27]. On the contrary, there are studies reporting that protein digestibility increases as the protein level increases in the diet [28]. It may be that the high digestibility of CP in the commercial native food contains fish meal as well as poultry meal as an animal protein source. Poultry meal has been used in other foods, but there is no fish meal. In various studies, CP digestibility in dog food ranges between 77.7-91.2% [20,22,23,25].

Dogs exhibited a clear preference for extruded food. One possible explanation for this difference could lie in the fact that pellets tended to crumble, forming flour that settles to the bottom of the container. Dogs were likely unable to consume this dusty residue, which remained in food pans. Moreover, the high heat in extruder processing increased flavour due to the Maillard reaction [3,6]. The enhanced taste and the uniform structure of extruded food appeared to facilitate increased feeding.

The faeces of dogs that consumed pellet food had a slightly softer consistency, probably due to the lower heat treatment during pelleting, which results in less starch gelatinization and decreased dry matter digestibility. However, faecal scores did not significantly differ across dog foods after controlling for the variation in consumption levels. Our observed values are within normal limits and similar to those reported by previous researchers [24,25,29].

In calculating the cost, it was determined that extruded food produced in this study could be 5 times more economical than imported food. According to the results of feeding experiments, extruded food can be easily recommended instead of imported food to dog owners.

We demonstrated that considerable variation exists in the protein, fat, ash, and energy content of animal and vegetable protein sources used to produce dog food. Thus, to ensure consistency in the nutritional value of dog food, raw feedstuffs should be analysed carefully before use.

Furthermore, although pelleted food is inexpensive, it is harder to digest than extruded food, while also being less preferred by dogs. Finally, the pellet form appears to be less efficient, given its propensity to be crumbled and leave behind powder that the animal cannot consume. This characteristic likely also causes nutrients to be lost from the diet.

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Research Article

Investigating the Bias Resulted from Ignoring Bulmer Effect on the Genetic and Economic Output in Progeny Test and Genomic Selection Program

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Abstract

This study aims to investigate the degree of bias resulted from ignoring Bulmer effect during the estimation of genetic and economic progress in progeny test and genomic selection programs. To this end, a deterministic approach based on gene flow method in a time horizon of 70 years was used. In this study, milk production was considered as the selection goal under a four-path selection strategy. In the progeny test, asymptotic genetic variance of sires and dams decreased by 67.59% and 64.97%, respectively. Also, in genomic selection program, asymptotic genetic variance in sires and dams decreased by 68.56% and 63.06%, respectively. The maximum reduction in genetic variance occurred in the first three generations. In the progeny test program, the bias of genetic progress per generation due to ignoring Bulmer effect was four times higher than genomic selection program, but this difference decreased significantly in the results of single round and continuous selection after 20 generations. Bulmer effect resulted in 51.64% and 44.62% reduction in the economic efficiency of progeny test and genomic selection, respectively. According to the results of this study, ignoring Bulmer effect in the investigations concerning comparison between progeny test and genomic selection seems to be unreasonable. Long-term selection has more severe effect on genetic and economic aspects of progeny test in comparison to genomic selection program via decreasing genetic variance.

Keywords: *Bulmer effect, Genomic selection, Bias, Progeny testing, Selection intensity*

Bulmer Etkisinin Gözardı Edilmesinin Projeni Testinde Genetik ve Ekonomik Sonuçları ve Genomik Seleksiyon Programındaki Olumsuz Etkisinin Araştırılması

Özet

Bu çalışmada Bulmer etkisinin göz ardı edilmesinin progeni testinde genetik ve ekonomik sonuçlarının tahmini ile genomik seleksiyon programındaki olumsuz etki derecesinin araştırılması amaçlanmıştır. Bu amaçla, 70 yıla yayılan gen akış metodu temelli belirleyici bir yaklaşım kullanıldı. Çalışmada, 4 yollu seleksiyon stratejisi altında seleksiyon hedefi olarak süt üretimi kullanıldı. Progeni testinde, baba ve annelerin asimtotik genetik varyansı sırasıyla %67.59 ve %64.97 azaltıldı. Genomik seleksiyon programında, baba ve annelerin asimtotik genetik varyansı da sırasıyla %68.56 ve %63.06 azaltıldı. Genetik varyansda maksimum azalma ilk üç nesilde gerçekleşti. Progeni test programında, Bulmer etkisini göz ardı etme sonucu, her nesildeki genetik ilerleme etkisi genomik seleksiyon programından dört kat daha fazlaydı. Ancak bu fark tek tur ve 20 nesil sonrasında devamlı seleksiyon sonuçlarında anlamlı derecede azaldı. Bulmer etkisi, progeni testinin ekonomik verimliliğinde ve genomik seleksiyonda sırasıyla %51.64 ve %44.62 azalmaya neden oldu. Bu çalışmanın sonuçları progeni testi ile genomik seleksiyonun karşılaştırıldığı incelemelerde Bulmer etkisinin göz ardı edilmesinin kabul edilebilir olmadığını göstermiştir. Uzun süreli seleksiyonun genetik varyansı azaltmak suretiyle genetik seleksiyon programı ile karşılaştırıldığında progeni testinin genetik ve ekonomik yönleri üzerine daha ciddi etkileri bulunmaktadır.

Anahtar sözcükler: *Bulmer etkisi, Genomik seleksiyon, Olumsuz Etki, Progeni testi, Seleksiyon yoğunluğu*

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INTRODUCTION

Due to the intensive use of artificial insemination and limited number of superior sires, the existing population of dairy cattle has limited size; its effective size is only 50-100 heads in each country [1]. Therefore, alleles of limited number of ancestors are shared among the population. Reduction in the size of selected population has serious effects on genetic variance of this population [1,2]. The selection not only influences the mean of population, but also affects the genetic variance. The decrease in the genetic variance could affect the economic-genetic progress of the subsequent generations [3]. Truncating a distribution affects both the mean and the variance of the distribution. The effect of selection on genetic variance was first investigated and involved in breeding estimations by Bulmer [4]. Hence, according to the investigations conducted by Bulmer, the effect is often referred to as the "Bulmer Effect".

The group of selected animals represents one tail of non-normal distribution curve and reduction factor of genetic variance depends on selection intensity. The genetic variance of a population prior to selection is partitioned into the parental and Mendelian sampling components. Only the parental contributions to genetic variance are affected by selection [4].

Utilization of IVF and MOET duplicates the effect of the limited number of selected superior individuals on genetic variance [5]. The highest selection intensity often belongs to sire of sire (SS) path. Dam of dam (DD) path has the lowest selection intensity due to high demand for replacement heifers in commercial dairy farms [6]. Correction for Bulmer effect has not been considered in many investigations carried out so far [7-11]. Moreover, nowadays, due to the need for decision-making in the selection of efficient animal breeding programs, many simulations are conducted in order to compare progeny test and genomic selection programs under a variety of strategies. The main point is that the variation in genetic variance using these two selection programs has remained unknown; different degrees of bias due to ignoring Bulmer effect may therefore lead to unreliable inferences in such comparisons. Thus, studying the bias rate of results due to ignoring Bulmer effect in progeny test and genomic selection seems necessary. In this study, the variation of genetic variance due to selection was calculated by simulating progeny test and genomic selection schemes during 20 generations. Then, based on these estimated variations in genetic variance, the genetic and economic progresses resulting from these two selection programs were calculated.

MATERIALS and METHODS

Description of Simulation Condition

A deterministic model based on gene flow method [12] was used to estimate the effect of altering the accuracy of genomic selection evaluations (AGSE) on economic efficiency (EE) of selection program through time horizon of 70 years. The time horizon of 70 years was chosen to ensure reaching the equilibrium phase for genetic growth [9]. All calculations were undertaken using MATLAB 8.0 programming language [13]. Population statistics, productive, reproductive and economic parameters were based on the Holstein cattle production in Iran. In the present study, the milk yield trait was considered as the selection goal, with genetic standard deviation of 477 kg and heritability of 0.29 based on average estimates offered by Chegini [14] and Ghavi Hossein-Zadeh [15] for Holstein dairy cattle in Iran. Productive and reproductive statistics of Iranian Holstein dairy cattle are shown in *Table 1*. EE was calculated as the ratio of the revenues to the costs of simulated programs.

Overall Structure of Progeny Test

A four-pathway -including sires of future sires (SS), sires of future dams (SD), dams of future sires (DS), and dams of future dams (DD) pathways- selection program was considered in the simulated progeny test program. The superior dams were selected from breeding population as DS. Also, a proportion of productive cows were inseminated by young bulls (test capacity) and the young bulls were evaluated using the daughters' records.

Revenues were divided into two parts: (1) revenues obtained from genetic progress and (2) non-genetic revenues. The revenues from genetic progress resulting from selection program depend on the economic value of milk trait; the non-genetic revenues, moreover, included the revenues from sale of omitted tested bulls or proven bulls. Generally, the costs were divided into three parts: quarantined costs, proving costs and the costs after proving. The proving costs (C_{prove}) were calculated as follows: (Formula1)

$$C_{prove} = N_{YB}\left(C_{YB} + (C_{cs} + C_{ps})S_{dose} + C_{In}S_{dose} + \sum_{t=1}^{t=w_{yr}} (C_m + C_f)\left(\frac{1}{1+i_c}\right)^t + (r_c n)\left(\frac{1}{1+i_c}\right)^{(1+w_{yr})}\right)$$

Where r_c is the recording cost per daughters, i_c discounting factor of cost, w_{yr} the number of expected years for testing the young bulls, C_f the feeding cost per young bull in a period of one year and C_m, the maintenance cost of each young bull in a period of one year. C_{YB} is the purchase cost of each young bull after quarantine, C_{cs} the collection cost of each sperm dose, C_{ps}, the producing cost of each sperm dose, S_{dose} the number of sperm doses resulting from young bulls for inseminating productive population in order to produce daughter progenies, C_{In} insemination cost, and R_C the recording cost of young bull's daughters. Considering the maintenance of proved sires for a period of 4 years, the costs after proving were calculated by the following (Formula 2):

$$CP_{m-f} = \sum_{t=5}^{t=8} ((C_c + C_{save} + C_m + C_f)(\frac{1}{1+i_c})^{w_{yr}+t})n_{SD}$$

Table 1. *Productive and reproductive parameters*

Items	Productive and Reproductive Parameters	Values units
Population parameters	Number of productive cows	502400
	Percentage of herd book population	33%
Biological variables	Heritability of milk production[1]	0.18-0.36
	Genetic variance of milk production	477 (kg)
	Proportion of captured genetic variance	0.875
	Twining rate	53.4 (%)
	Abortion rate	62.8 (%)
	Number of needed mothers for producing one YB	6.07
	Alive daughters per cow	40 (%)
	Young cows under milk recording as DS	30 (%)
	Death birth rate	29.3 (%)
	Death rate at 3 month	27.8 (%)
	Insemination number per pregnancy	2.22
Technical variables	Years of using a proved bull	4
	Quarantine time	6 (month)
	Time interval between 2 recalculating haplotype effects	2
	Number of individual in training set[2]	500-10000
	Number of daughters per bulls in training set	100
	First calving age	2.13
	Open days	397.8
	Sperm production per bull	18000 (dose)
Economic variables	Insemination cost	7 (US$)
	Cost of buying a YB	1429 (US$)
	Maintenance and feeding cost in Quarantine/month/YB	157 (US$)
	Cost of testing for diseases in Quarantine/YB	28 (US$)
	Cost of collecting each Vial of Sperm	2 (US$)
	Cost of recording/daughter	2 (US$)
	Feeding cost/year/YB	1543 (US$)
	Maintenance cost/year/YB	343 (US$)
	Price of culled bull	2000 (US$)
	Genotyping cost[3]	50-400 (US$)
	Discount rate of costs	0.06
	Discount rate of returns	0.08
	Economic value of milk production (US$)	0.23

1: *In the basic scenario number of individuals in training set was 1.000, in the first scenario varied from 500 to 3.500 and in order to produce varied from 500 to 10.000;* ***2:*** *In the basic scenario the assumed heritability was 0.29 and in the second scenario varied from 0.18 to 0.36;* ***3:*** *In the basic scenario genotyping cost was 100 US$ and in the third scenario varied from 50 to 400 US$*

C_c is the collection cost of 18000 sperm dose, C_{save} the costs of production and storing of 18000 sperm dose, C_m the maintenance cost of each proved sire, and C_f the annual feeding cost of each proved sire. Finally, the sum of these costs was considered as the costs resulting from single round selection based on progeny test.

Structure of Genomic Selection Program

In genomic selection program, all productive cows were inseminated by young bulls, but, in progeny testing program, a proportion of productive cows (P: test capacity) was inseminated by young bulls and the remaining ones were inseminated by proven bulls. In this investigation we tried to consider the required and available numbers of individuals in different paths of genomic selection program, close to that of progeny test program. The accuracy of genetic evaluations was calculated using (Formula 3) [16].

$$r = w\sqrt{\frac{N_p R^2}{N_p R^2 + Me}}$$

Where w shows the proportion of genetic variance captured by markers, and was assumed to be 0.875 [16]; N_p is the number of individuals in the training set and R^2 the reliability of breeding values in the sire's population. R^2 was calculated by; $\frac{n}{\frac{4-h^2}{h2}+n}$

n is the number of daughters of each progeny tested bulls. Also, M_e is the number of independent genome segments. In all paths, except DD, selection was implemented based on genomic information. Accuracy of DD path was assumed to be the square root of heritability.

Estimated effective number (Ne) of Holstein population in Iran is approximately 62.49; based on this Ne, the calculated Me is 440.07 [17].

We planned the re-calculation of haplotype effects in the training set to be conducted every two years. Hence, it is necessary to keep up recording of sire's daughters in the training set. The cost of proving was calculated as follows: (Formula 4)

$$C_{prove} = N_{YB}(C_{YB} + C_{f-m} + R_c) + (N_{YB} + rr_{DS}N_{DS} + \frac{N_p}{h})G_c$$

Where G_c is the genotyping cost per selection candidate, h the time interval to determine Haplotype effects in training set and G_{cost} the costs of genotyping. The after proving costs were also calculated in a way similar to that of progeny test, except that the 1 to 5 year-old sires were used for insemination of productive population instead of 5 to 8 year-old proven bulls (in progeny test) and the discounting rates regarding these years were used. As well, in this selection method all costs were summated at the end of calculations in order to calculate the cost of performing single round selection.

Consideration of Bulmer Effect in Two Selection Programs

The value of genetic variance affected by selection (Bulmer

effect) was calculated using the method represented by Bulmer [4]. In four-path selection, genetic variance of sires and dams are calculated separately due to the difference in how the parents are selected for producing replacement sires and dams [18]. Hence, the genetic variance in generation t, in sire ($\sigma^2_{S^*}$)and dam ($\sigma^2_{D^*}$) populations was calculated according to the following formulas (5-7) [18]: (Formula 5)

$$\sigma^2_{S^*} = 0.25\left(1 - K_{SS}r^2_{SS^*}\right)\sigma^2_{S(t-1)} + 0.25\left(1 - K_{DS}r^2_{DS^*}\right)\sigma^2_{D(t-1)} + 0.5\sigma^2_0$$

Where $\sigma^2_{S(t-1)}$is sire genetic variance in generation t-1, $\sigma^2_{D(t-1)}$ dam genetic variance in generation t-1, K_{SS} the factor by which the variance in SS path is reduced, K_{DS}reduction factor of genetic variance in DS path, $r^2_{SS^*}$ the accuracy of selection in SS path, $r^2_{DS^*}$ the selection accuracy in DS path, and σ^2_0 the variance due to Mendelian sampling. (Formula 6)

$$\sigma^2_{D^*} = 0.25\left(1 - K_{SD}r^2_{SD^*}\right)\sigma^2_{S(t-1)} + 0.25\left(1 - K_{DD}r^2_{DD^*}\right)\sigma^2_{D(t-1)} + 0.5\sigma^2_0$$

Where K_{SD} is the reduction factor of genetic variance in SD path, K_{DD} the reduction factor of genetic variance in DD path, $r^2_{SD^*}$ the accuracy of selection in generation t in SD path, and $r^2_{DD^*}$ the accuracy of selection in generation t in DD path. Therefore, genetic variance is reduced by factor K which in each selection path (K_{ii}) depends on selection intensity (i_{ii}) and truncation point (X_{ii}) of the path. Given the truncation selection, K is calculated by (Formula 7) [4]:

$$K_{ii} = i_{ii}\left(i_{ii} - X_{ii}\right)$$

Comparison of Two Selection Programs

In both selection programs, genetic progress per generation was calculated by multiplying the selection intensity, the accuracy of each path and genetic standard deviation of milk trait and genetic progress results from single round and continuous selection, it is the gene flow that estimated by tracking the flow of genes in population through 70- yeartime horizon by the use of gene flow method. When it comes to generations, genetic progress is obtained from single round and continuous selection but in the 70-year time horizon it is obtained using gene flow method. At first, the genetic and economic aspects were investigated during 70 years without considering Bulmer effect and thereafter; all output calculations were conducted by correction for Bulmer effect. The bias was calculated by dividing the corrected value by non-corrected value. The economic efficiency (EE) was obtained as the ratio of total discounted return to total discounted costs.

RESULTS

Genetic variance and heritability of milk production in sire and dam pathways in both cases of taking into account and ignoring the Bulmer effect (during 20 generations of continuous selection) in progeny test and genomic selection program are represented in *Table* 2. At the asymptotic point, genetic variance of sires and dams decreased by 67.59% and 64.97% and reached 73753.01 and 79695.13 (kg^2), respectively. The trend of genetic variance in sires and dams in the progeny test program is represented in *Fig. 1*. According to this diagram, the maximum reduction in genetic variance of sires and dams occurred in the first three generations and then the slope of variance reduction decreased by time and reached its asymptotic value in generations 14 and 15 in sire and dam pathways, respectively. Hence, in the first three generations after starting selection, a considerable upstream bias would occur in genetic variance due to ignoring the Bulmer effect. Because of higher selection intensities in paths DS and SS compared to that in paths DD and SD, a further reduction in genetic variance of sires was observed. Also, in genomic selection program, the genetic variance in sires and dams reached its asymptotic phase after 12 and 16 generations, respectively. In asymptotic phase, the genetic variance of sires and dams decreased by 68.56% and 63.06% and reached 71532.06 and 84045.22 (kg^2), respectively. *Fig.* 2 shows the trend of genetic variance in selection program based on genomic information. The trend of genetic variance in sires and dams in genomic selection method was similar to progeny test. Also the maximum reduction of genetic standard deviation in genomic selection occurred in the first three generations and due to higher selection intensities in paths DS and SS, the dams resulting from these two paths experienced more reduction in genetic variance. The percentage of reduction in asymptotic genetic variance in sires and dams were approximately similar in both selection methods. The average generation interval through all paths in the progeny test method and genomic selection were 5.29 and 3.25 years, respectively. Hence, because of different generation interval in these selection programs, comparison of the annual variation in genetic

Table 2. *Genetic variance and heritability in sire and dam pathways in both cases of ignorance and consideration of Bulmer effect (during 20 generations of continuous selection) in progeny test and genomic selection program*

Selection Program	Sire Genetic Variance	Dam Genetic Variance	Sire Heritability	Dam Heritability
PT	227529	227529	0.29	0.29
PT_b	73753.01	79695.13	0.1169	0.1252
GS	227529	227529	0.29	0.29
GS_b	71532.06	84045.21	0.11	0.13

PT= *Progeny test program,* ***PT_b***= *Progeny test in the case of adjustment for Bulmer effect,* ***GS***= *Genomic selection,* ***GS_b***= *Genomic selection in the case of adjustment for Bulmer effect*

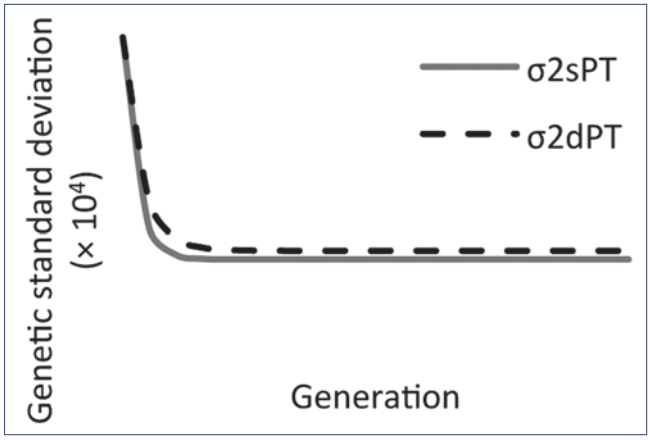

Fig 1. The trend of genetic variance in sires (σ2sPT) and dams (σ2dPT) in progeny test

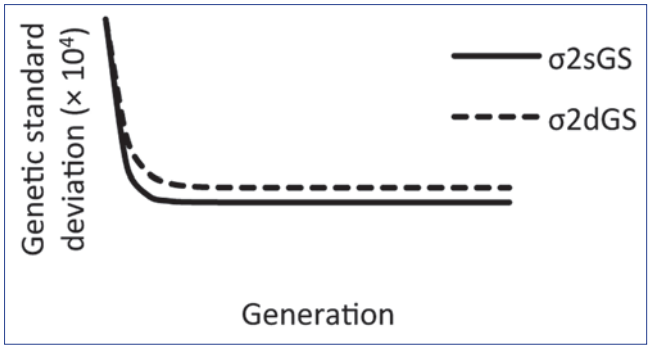

Fig 2. The trend of genetic variance in sires (σ2sGS) and dams σ2dGS) in genomic selection

variance through 20 generations is not reasonable. The primary value of heritability in both selection methods was assumed to be 29%. According to *Table 2*, in the progeny test, heritability in sires and dams decreased by 59.69% and 56.83% and reached 0.1169 and 0.1252, respectively. The heritability variations were equal to the variations of genetic variance. According to *Table 3*, the heritability of sires and dams in genomic selection method was decreased by 60.76% and 54.79%, respectively.

The asymptotic values of heritability in sire and dam pathways were 0.11 and 0.13 (kg^2), respectively. The reduction of genetic variance caused by continuous selection in progeny test resulted in the reduction of the accuracy of genetic evaluations in all the paths. The accuracy of genetic evaluations in paths SD and SS was decreased by 7.99%. The reduction in the accuracy of paths DD and DS was estimated about 34.30% (data not shown). After correction for Bulmer effect in genomic selection, the estimated accuracy of sires in training set changed from 0.8866 to 0.7534 (equal to 14.91%). As a result of the reduction in the accuracy of breeding evaluation of sires in training set, the accuracy of genetic evaluations in paths SS, SD and DS decreased by 3.01%. The reduction in the accuracy of path DD was 32.76%, which was almost

Table 3. *Genetic progress per generation (per genetic standard deviation) in sires of future sires (SS), sires of future dams (SD) dams of future sires (DS) and dams of future dams (DD) in the progeny test and genomic selection programs in both cases of ignoring and considering the Bulmer effect*

Selection Program	SS	SD	DS	DD
PT	2.23	1.24	1.39	0.19
PT_b	2.05	1.15	0.91	0.12
GS	1.11	1.11	1.84	0.19
GS_b	1.08	1.07	1.78	0.13

PT*= Progeny test program,* ***PT_b*** *= Progeny test in the case of adjustment for Bulmer effect,* ***GS****= Genomic selection,* ***GS_b*** *= Genomic selection in the case of adjustment for Bulmer effect*

the same as the reduction in the accuracy of this path in the progeny test program. Considering the results of this study, in the paths where the selection was based on the genomic information, the accuracy of breeding evaluations was less influenced by Bulmer effect. In path DD, in which the selection was not based on the genomic information, the reduction in the accuracy was higher. In this path the accuracy rapidly decreased in the first three generations and then slight variations were observed until it reached the asymptotic phase. Genetic progress per generation (per genetic standard deviation) in sires of future sires (SS), sires of future dams (SD), dams of future sires (DS) and dams of future dams (DD) in the progeny test and genomic selection programs in both cases of ignoring and considering the Bulmer effect are shown in *Table 3*. In progeny test program in the case of ignoring Bulmer effect, the biases in genetic progress in SS, DD, DS, and DD paths were equal to 8.15%, 7.56%, 34.30% and 34.05%, respectively. In progeny test program, due to higher reduction in the accuracy of breeding evaluations of dams, the maximum reduction in genetic progress per generation was observed in DS and DD paths. Because of higher selection intensity in path DS, Bulmer effect caused higher reduction in genetic progress per generation in this path. In genomic selection method, in the case of ignoring the Bulmer effect, the biases in genetic progress in paths SS, SD, DS, and DD were about 3.07%, 3.07%, 3.99% and 31.89%. In this selection method, due to higher percentage of reduction in the accuracy of DD path, more depression in genetic progress occurred. Genetic progress per generation (G), genetic progress resulting from single round selection after 70 years (G_O) and continuous selection for 70 years (G_C) in all paths of selection program based on the progeny test and genomic information in both cases of ignoring and considering Bulmer effect are presented in *Table 4*. In progeny test program, ignoring the Bulmer effect resulted in 16.24% upward bias in the genetic progress per generation. The biases resulted from single round and continuous selections were equal to 51.81% and 51.97%, respectively. In genomic selection, ignoring the Bulmer effect caused 4.48 percent upward bias in the genetic progress per generation. In spite of small bias in estimated

Table 4. *Genetic progress per generation (G), genetic progress resulting from single round selection after 70 years (G_o) and continuous selection after 70 years (G_c) in all paths of selection program based on the progeny test and genomic information in both cases of ignoring and considering Bulmer effect*

Selection Program	G	G_o	G_c
PT	5.05	113.76	0.02
PT_b	4.23	54.82	0.07
GS	4.24	155.60	0.02
GS_b	4.05	87.79	0.01

***PT**= Progeny test program, **PT_b**= Progeny test in the case of adjustment for Bulmer effect, **GS**= Genomic selection, **GS_b**= Genomic selection in the case of adjustment for Bulmer effect*

***Table 5.** Discounted cumulative profit and economic efficiency after 70 years continuous selection based on progeny test and genomic information in both cases of ignoring and considering Bulmer effect*

Selection Program	Discounted Cumulative Profit	Economic Efficiency
PT	1835	11.00
PT_b	793	5.32
GS	3402	23.89
GS_b	1817	13.23

***PT**= Progeny test program, **PT_b**= Progeny test in the case of adjustment for Bulmer effect, **GS**= Genomic selection, **GS_b**= Genomic selection in the case of adjustment for Bulmer effect*

genetic progress per generation, short generation interval in genomic selection led to higher bias in genetic progress through single round and continuous selection in 70 years, but still it was less than in progeny test *(Table 5)*.

DISCUSSION

In the progeny test program, the bias of genetic progress per generation in the case of ignoring Bulmer effect was four times higher than genomic selection program, while, this difference significantly decreased in the results of selection after 70 years.

Discounted cumulative profit and economic efficiency after the 70=year continuous selection, based on progeny test and genomic information, in both cases of ignoring and considering Bulmer effect is presented in *Table 5*. In progeny test program, discounted profit per dairy cattle in the case of adjustment for Bulmer effect was 1042$ less than the non-corrected case (equal to 56.79 percent upstream bias in the case of ignoring Bulmer effect). As mentioned previously, the bias of genetic variance in the sire and dam paths was about 67.59% and 64.97%, respectively. The bias percentage in discounted profit was less than that of genetic variance in sires and dams. Correction for Bulmer effect induced 46.59% reduction in discounted cumulative profit per dairy cattle in genomic selection program *(Table 5)*. According to these results, reduction of profit due to the reduction of genetic variance in progeny test is more serious than in genomic selection. Bulmer effect resulted in 51.64% reduction in economic efficiency of progeny test. In genomic selection, reduction of variance resulted in 44.62% decrease in the economic efficiency. Bijma [19] explained that ignoring Bulmer effect, leads to over-estimation of response and accuracy of selection.

The results of the current research showed that the overestimation is different for genomic selection and the progeny test. Therefore, ignoring Bulmer effect in the investigations concerning comparison between progeny test and genomic selection (e.g.[9,20-24]) seems to be unreasonable. The bias in the results of such studies might influence the confidentiality of related results and inferences. Schaeffer [11] studied the advantages of selecting individuals based on genomic information, and compared it with conventional progeny test. The influence of Bulmer effect on the genetic variance and asymptotic genetic progress in long term was ignored in this study. König et al.[20] explained that it is not probable to involve Bulmer effect in deterministic simulations concerning selection strategies. Thus, this effect was ignored in their calculations and comparisons. In their study, the progeny test was compared with different strategies of genomic selection. Different levels of accuracies and selection intensities were assumed in their simulations. According to the results of this study, the Bulmer effect is expected to differently influence the results of these comparisons.

The results of the current research showed that the effect of genetic variance reduction, caused by long-term continuous selection, on the economic factors is stronger in progeny test than in genomic selection and would reduce economic factors more rapidly. Börner and Reinsch [22] compared a progeny test program with different strategies of genomic selection. They assumed that both selection programs would affect the genetic variance in the same way. They suggested that ignoring this effect does not make any changes in the comparison of genomic selection and progeny test because the effect is equally ignored in both programs. According to the results of the current, Bulmer effect differently influences the result of selection, depending on the selection intensity and the type of selection program; therefore, error may occur in the comparisons of these two selection programs.

Through the last decade, genomic selection based on low and high density markers [25,26] were applied for studying genetic variation and structure of domestic animals populations [27-29]. Hence, utilization of genetic information allows for the selection of animals which induce more genetic variation. According to the aspects mentioned above and also the results of the current study, the utilization of genomic information could significantly prevent the reduction of genetic variation and its side effects. Genomic prediction has successfully been tested

in large breeds of dairy cattle [30,31]. Other applications of genomic information in the management of dairy cattle include estimation of family relationship and inbreeding coefficient using single-nucleotide polymorphism [1,31], homozygosity values [32] or combination of different information resources [33]. In genomic selection, the inbreeding rate can be much lower than in traditional BLUP or mass selection because Mendelian sampling effects can be estimated more accurately in genomic predictions, which leads to better differentiation within families and reduce the co-selection of sibs [34,35].

However, the accuracy of genomic EBV has a diminishing-return relationship with the size of the reference population. As a consequence, when GS schemes have a moderate decrease in generation interval, relatively small reference population sizes are needed to obtain a response equal to that with selection on traditional BLUP-EBV based on own performance or progeny information. Thus, when the trait of interest cannot be recorded on the selection candidate, GS schemes are very attractive, even when the number of phenotypic records is limited, because traditional breeding schemes would have to rely on information from relatives with many phenotypic records and long generation intervals in the case of progeny testing.

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Short Communication

Association of Single Nucleotide Polymorphism in *Melanocortin Receptor* Gene with Egg Production Traits in Lohmann Brown Chickens

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Abstract

Melanocortin receptor (MC4R) plays an important role in central melanocortin system and regulation of feed intake in birds. In this study the melanocortin gene (MC4R) was proposed as a candidate gene for egg production traits (yield and weight) in Lohmann Brown hens. DNA from blood samples was extracted to amplify the MC4R gene and the purified PCR products were sequenced. Alignment of sequence data from each group revealed that there is a variation detected in MC4R-1 at nucleotide 22 (T-G) (sense mutation) for high egg weight. Hens with the AB genotype produced significantly higher egg weight compared to hens with the AA genotype. There was no significant effect of this mutation on egg yield. The detected mutation and the analysis of egg production means revealed a significant association between MC4R polymorphism and egg weight. The MC4R-SNP could be considered as a useful marker in chicken selection especially for egg weight.

Keywords: *Lohmann Brown, MC4R, Egg weight, SNP, Sense mutation*

Lohmann Kahverengi Tavuklarda *Melanocortin Reseptörü* Geninde Tek Nükleotid Polimorfizmi İle Yumurta Verim Özellikleri Arasındaki İlişki

Özet

Melanocortin reseptörü (MC4R) merkezi melanocortin sisteminde ve kuşlarda yem tüketiminin düzenlenmesinde önemli rol oynar. Bu çalışmada melanocortin geninin (MC4R) Lohmann Kahverengi tavuklarda yumurta verim özellikleri (sayı ve kilo) üzerine etkili bir gen olabileceği düşünülmüştür. MC4R genini amplifiye etmek amacıyla kan örneklerinden DNA ekstrakte edildi ve PCR ürünlerinin sekanslaması yapıldı. Her bir grupta sekans verisinin dizilimi MC4R-1'in nükleotid 22'sinde (T-G) (Sense mutasyon) yüksek yumurta ağırlığı için varyasyon bulunduğunu ortaya koydu. AB genotipine sahip tavuklar AA genotipine sahip tavuklar ile karşılaştırıldığında istatistiki olarak daha yüksek yumurta ağırlığına sahiptiler. Bu mutasyonun yumurta sayısı üzerine bir etkisi bulunmamaktaydı. Tespit edilen mutasyon ve ortalama yumurta üretimi analizi MC4R polimorfizmi ile yumurta ağırlığı arasında anlamlı bir ilgi bulunduğunu gösterdi. MC4R-SNP özellikle yumurta ağırlığı bakımından önemli bir belirteç olarak kullanılabilir.

Anahtar sözcükler: *Lohmann Kahverengi, MC4R, Yumurta ağırlığı, SNP, Sense mutasyon*

INTRODUCTION

Understanding the associations between genotype and phenotype is very important for faster improvement in animal breeding with potential economic benefits. Results inferred from molecular genetic studies play an important role in breeding value prediction systems and in the construction of commercial lines and populations. In the 20th century, strong selection of production traits started when commercial breeds were selected for egg and meat production [1]. Selection programs based on productive traits have been of major importance to the poultry industry. Amie-Marini et al.[2] reported that single nucleotide polymorphism (SNP) is an effective method to detect nucleotide sequence mutation in amplified DNA. The investigation strategy for a specific favorable SNP involves a novel and lengthy process of the identification of the DNA molecular marker for a major effect gene. Holsinger and Weir [3] revealed the importance of discovery a large number of SNPs in the genomes from several species that has enabled exploration of genome-wide signatures in selection via an assessment of variation in marker allele frequencies

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among these populations. Genes associated with productive traits have been identified using single nucleotide polymorphisms of many candidate genes [4,5]. It is recognized that the egg production traits of chickens are controlled by a complicated multiple genes [6].

Melanocortin 4 receptor (MC4R) is a protein expressed in the hypothalamus in humans and it has been found to be involved in feed intake, the regulation of metabolism and body weight [7]. Mutations of the MC4R gene were associated with the appetite and growth in many animal species [8]. According to the results of El-Sabrout [9] on rabbit, MC4R gene has many important behavioral and growth functions. Moreover, the mutations of MC4R gene have been found association with carcass quality in cattle [10], and broiler chickens [11].

Few researches have been published to improve the effect of MC4R mutations on egg production traits in chickens. Therefore, the present study was carried out to investigate the association between MC4R gene and the egg production traits (yield and weight) in Lohmann Brown chickens.

MATERIAL and METHODS

Animal, Housing and Feeding Management

This experiment was carried out on 200 Lohmann Brown hens (11 months of age). Hens were divided into two groups according to their egg weight (120 hens for control egg weight (AA avg. 57.7 g), 70 hens for high egg weight (AB avg. 60.4 g) and 10 hens for low egg weight (BB avg. 55.1 g)). Birds were housed in single cages with an intensive system and were offered *ad libitum* access to fed commercial pelleted diet (18% protein and 2800 Kcal/kg). The study was approved by Alexandria University Animal Ethics Committee (2016).

Molecular Analysis

DNA was extracted from whole blood by taking random blood samples (20 samples per group) from the wing vein in centrifuge tubes containing EDTA as anticoagulant using DNA isolation kit (Zymo® Research, USA) following the manufacturer's protocol and stored at -20°C until used. Two different primers from MC4R gene: MC4R-1 (500 bp) and MC4R-2 (492 bp) [12] were investigate from Biosearch Technologies (USA) to generate PCR profiles from DNA samples *(Table 1)*. The total PCR reaction volume was 25 µL, included 3 µL of genomic DNA of each group, 1 µL of each primer, 15 µL of 2× Thermo Multiplex PCR Master Mix and 6 µL of RNase-free water. PCR program was performed using a thermal cycler (Thermo® Scientific Corporation, EU) included three main steps: initial denaturation at 95°C for 5 min, followed by 35 cycles, denaturation at 95°C for 30 sec; annealing at 45°C for 30 sec and lasted by extension at 72°C for 1 min then an extension cycle at 72°C for 8 min. Amplicones were separated on 1.5% agarose gel, stained with Ethidium Bromide and visualized under UV Transilluminator. GelAnalyzer application [13] was used to analysis the determined DNA bands on the agarose gel. The amplified DNA fragments of growth genes were digested with *MSP1*. The RFLP was carried out on PCR product according to Zhou et al.[14]. The purified DNA was sequenced using the automated sequencer by Macrogene Company (South Korea). Sequence analysis and alignment were carried out using the CodonCode Aligner software (http:// www.codoncode.com/aligner).

Table 1. *List of the PCR primers used in this study and their sequences*

Primer Code	Nucleotide Sequence (5'-3')
MC4R-1	F 5'- CAACCCCAGTTACCAGCACT-3' R 5'- GCATTGCTGTGCAGTCCATA-3'
MC4R-2	F 5'- CCATTGCAGTGGACAGGTATT-3' R 5'- TCCGGAGTGCATAAATGAGA-3'

Egg Production Traits

Data of egg yield (%) and egg weight (g) (n=50 samples per group) were recorded daily during the experiment period (6 months). Egg weight of all eggs was determined with digital balance (g).

Statistical Analysis

Statistics of means differences in egg and weight production between the two groups were determined by ANOVA followed by Duncan´s multiple range test using SPSS [15]. The association between the genotypes of MC4R gene and the egg production traits were analyzed by the means and standard errors method as applied in the General Linear Model (GLM) procedure of SPSS [15] according to the following statistical model:

$$Y_{ijm} = \mu + G_i + EY_j + e_{ijm}$$

Where Y is the dependent variable (egg weight), µ is the overall mean of observations, G is the fixed genotype effect, EY (egg yield) is the covariate, and e is the residual error.

RESULTS

All primers were amplified and yielded distinct polymorphic PCR profiles at molecular weight ranged from 210 to 370 bp. RFLP analysis of PCR product using *MSP1* did not produce restriction fragments. The results of MC4R-1 electrophoresis showed three genotypes (AA, AB and BB) with frequencies of 0.60, 0.35 and 0.05, respectively *(Table 2)*. The frequency of allele A was 0.78 while the frequency of allele B was 0.22. It means that allele A was dominant in Lohmann Brown hens. The purified PCR products were sequenced in those had the highest and lowest egg weight. Alignment of sequence data of 15 samples from

Table 2. *Genotype and allele frequencies of MC4R-1 in Lohmann Brown*

Number of Hens	Genotype Frequency (n)			Allele Frequency	
	AA	AB	BB	A	B
200	0.60 (120)	0.35 (70)	0.05 (10)	0.78	0.22

Table 3. *Means and standard errors (Mean± SEM) of egg yield and egg weight for Lohmann Brown*

Genotypes	N	Egg Yield (%)	Egg Weight (g)
AA	120	87.02±1.72	57.70±0.41^{b}
AB	70	86.65±1.61	60.43±0.38^{a}
BB	10	87.40±1.33	55.10±0.30^{c}
Significance	-	NS	*

*^{a,b,c}Means in the same column with different superscripts are significantly different (P<0.05). **NS**= not significant at (P<0.05); **AA** = control egg weight hens; **AB** = high egg weight hens; BB = low egg weight hens*

Table 4.*Association between MC4R SNP and egg weight in Lohmann Brown*

SNPs	Trait	Genotypes		P-value
		AA	AB	
MC4R-1	Egg weight (g)	57.70±0.41^{b}	60.43±0.38^{a}	0.03

Values are presented by the means and standard errors (Mean ± SEM); ^{a,b}Means in the same row with different superscripts are significantly different (P<0.05)

each group revealed that there is a mutation detected in MC4R-1 at nucleotide 22 (T-G) (sense mutation) for high egg weight, while there was no variation detected in MC4R-2. Means and standard errors (Mean±SEM) of egg yield and egg weight shown in *Table 3*. AB genotype had significantly (P<0.05) higher egg weight compared with AA. On the other hand, there was no significant difference detected between the two groups in egg yield. MC4R gene showed significant (P<0.05) association with egg weight in Lohmann strain *(Table 4)*. The results of SNP polymorphisms demonstrate the possibility to detect association between egg weight in Lohmann chickens and the efficiency of the used primers to predict through the genetic specificity using the single nucleotide polymorphism of MC4R.

DISCUSSION

Many previous studies were interested to investigate the relationship between the melanocortin-4 receptor gene (MC4R) and the body weight of animal. To determine whether there was an association of MC4R polymorphism with egg production traits in chickens, this study was carried to test two parts of MC4R gene (MC4R-1, MC4R-2) as candidate gene for Lohmann Brown hens (commercial layers strain). The frequency of heterozygous genotype (AB) has higher egg weight compared to homozygous (AA) and (BB) genotypes. Also, one sense mutation was identified in high egg weight group (AB). Therefore, it may be assumed that the MC4R-1 gene affected egg weight by regulating of appetite of hens. Analyses of MC4R SNPs and egg weight records showed significant association of MC4R genotypes with egg weight *(Table 4)*.

Davies et al.[9] reported that sense mutation can change the gene expression, which in turn a different protein with different characterizes is created as a result of amino acids change. This protein may lose its function or become activated or exhibit a new function. It is possible that the variation happened in amino acids due to the MC4R mutations causes a significant change of the MC4R function. Amino acids change may also affect the biosynthesis of other nutrients. It can stimulate the feed intake, metabolism and growth of egg, which in turn affect the egg weight. This finding is in agreement with El-Sabrout and Aggag [17], who found that MC4R plays an area responsible for controlling feed intake behavior, which in turn affect the body weight.

In addition, the effect of MC4R in chicken's egg weight suggests it may be an important genetic marker for the production-related traits. The hens within hetero-zygous genotype (AB) at the MC4R gene loci had superior egg production traits. This finding is useful to get commercial egg production chickens with superior production traits. Therefore, this study aims also to enhance selection efficiency on hen productive performance. The use of marker-assisted selection can augmented the efficient genetic improvement in these quantitative traits [18]. Moreover, MC4R SNPs located at candidate genes for economic traits allow prediction of the genetic merit of individuals and combined with guarantee consumer protection.

According to the results of this study, there is a significant association between MC4R gene polymorphism and egg weight in Lohmann Brown chickens. The results of single nucleotide polymorphism demonstrate the efficiency of used associated genes to predict through the genetic specificity. The results are also effective in chicken selection for high egg weight without affecting the egg yield. MC4R-1 SNP was potential useful DNA marker for selecting excellent individuals in marker-assist selection (MAS) breeding in relation to egg production traits in chickens. Further studies through expanded and different sampling with more details under various molecular levels will be required to provide clearer explanations.

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Case Report

Pregnancy Loss due to Partial Hydatidiform Mole in a Cat [1]

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[1] *This report was orally presented at 5th Veterinary Obstetrics and Gynaecology Congress which was held in Antalya/ Turkey, 2013*

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Abstract

Molar changes of the placenta are exceptionally rare in animals and in the cat, only one case of partial hydatidiform mole in a stillborn kitten was reported. A 3-year-old female cat was referred for anorexia, vomiting, depression and vaginal discharge. Abdominal distention and pain were noted. Blood count abnormalities were also observed in the cat. A septated, enlarged uterus with anechogenic content was diagnosed on ultrasonographic examination. Ventral midline ovariohysterectomy was performed. An uneventful recovery was observed and total blood count was within normal range on day 3 following ovariohysterectomy. Partial hydatidiform mole diagnosis was made based on the presence of embryos, gross appearance and histopathological findings. This report reflects the clinical presentation and histopathological findings of a partial hydatidiform mole leading to pregnancy loss in the cat.

Keywords: *Feline, Partial hydatidiform mole*

Bir Kedide Kısmi Hidatidiform Mol Nedeniyle Gebelik Kaybı

Özet

Hayvanlarda plasentanın molar değişiklikleri son derece nadir olup kedilerde yalnızca ölü doğan bir yavruda kısmi hidatidiform mol olgusu bildirilmiştir. 3 yaşlı dişi bir kedi iştahsızlık, kusma, durgunluk ve vaginal akıntı şikayetleri ile getirildi. Abdominal gerginlik ve ağrı kaydedildi. Kedide ayrıca kan sayımı anormallikleri gözlendi. Ultrasonografik muayenede anekojenik içeriğe sahip, septumlu, genişlemiş uterus belirlendi. Ventral orta hattan ovaryohisterektomi yapıldı. Hızlı bir iyileşme gözlendi ve ovaryohisterektomiyi takiben 3. günde tam kan sayımı değerleri normale döndü. Embriyoların bulunması, makroskobik görünüm ve histopatolojik bulgulara dayanılarak kısmi hidatidiform mol tanısı konuldu. Bu raporda, bir kedide gebelik kaybı ile sonuçlanan kısmi hidatidiform mol olgusunun klinik görünümü ve histopatolojik bulguları tanımlanmıştır.

Anahtar sözcükler: *Kedi, Kısmi hidatidiform mol*

INTRODUCTION

Hydatidiform mole is a trophoblastic lesion characterized by a hydropic (ie, vacuolar) swelling of the chorionic villi and trophoblastic proliferation. It starts at the time of fertilization due to a defective union of the sperm and ovum, which causes an aberrant proliferation of trophoblastic tissue. Fluid filled and edematous placental villi are observed as grape-like structures. In the human, a molar pregnancy is defined as complete or partial depending on the way of formation and differences in histopathological features and karyotype. A complete mole is usually formed by union of anuclear ovum and a haploid (23X) spermatozoon, which then undergoes duplication [1]. The resultant tissue is entirely paternal in origin, usually with a 46XX karyotype. A few of complete moles arise from fertilization of empty ovum by two spermatozoa and they have a 46XY or 46XX karyotype [2]. Primary histopathological features of complete moles are significant atypia of trophoblastic cells and generalized trophoblastic hyperplasia and hydropic swelling. Fetal tissue is not identified in complete molar pregnancy [3].

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A partial mole is the result of fertilization of ovum by two spermatozoa or by one spermatozoon which reduplicates itself. Consequent genotypes are 69.XXY (triploid) or 92.XXXY (tetraploid), respectively [4]. The trophoblastic cells at the implantation site show mild atypia and only diffuse areas of trophoblastic hyperplasia and swelling are observed. Fetal tissue is identified in partial moles. A fetus that develops in a partial molar pregnancy is nonviable and exhibits congenital anomalies associated with triploidy [3].

Hydatidiform moles are rarely observed in animals and most of the reported cases are in cows [5-7]. In cats, only one partial hydatidiform mole in a stillborn kitten was reported [8]. The aim of this study was to describe the clinical presentation and histopathological findings of partial molar pregnancy in a cat.

CASE HISTORY

A 3.2 kg, 3-year old, living indoor/outdoor female cat was referred with a history of anorexia, vomiting, depression and vaginal discharge for 3 days. On clinical examination, rectal temperature was 38.2°C and small amount of sero-sanguineous vaginal discharge was observed. Abdominal distention and pain were noted on abdominal palpation. Complete blood count (Exigo EOS Vet Analyzer, Sweden) revealed mild lymphocytosis (7.8×10^9/L; reference values: 1.0-7.0×10^9/L), monocytosis (1.1×10^9/L; reference values: 0.2-1.0×10^9/L), increased mean corpuscular hemoglobin concentration (39.3 g/dL; reference values: 31.0-38.5 g/dL) and thrombocytopenia (188×10^9/L; reference values: 200-500×10^9/L). Serum biochemistry (Erba XL 600, Mannheim, India) revealed no abnormalities. A septated, enlarged uterus with anechogenic content was diagnosed by ultrasonography (ProSound II 5 MHz sector; Aloka, Tokyo, Japan). No embryonic/fetal structure was observed *(Fig. 1A)*. Serum hormone concentrations were determined by electrochemi-luminescence immunoassay using an autoanalyser (Cobas6000 C601; Roche Diagnostics, Mannheim, Germany). Peripheral blood levels of estradiol, progesterone, follicle stimulating hormone and testosterone were <5.00 pg/mL, 2.92 ng/mL, <0.10 mIU/mL and 0.03 ng/mL, respectively. Ventral midline ovariohysterectomy was performed under general anesthesia. Anesthesia was induced with propofol (2% propofol, Fresenius Kabi GmbH, Austria) at approximately 6 mg/kg/minute intravenously and maintained with isoflurane (Isoflurane USP® Adeka, Turkey) delivered in 100% oxygen at a flow rate of 500 mL/kg/min through an Ayre›s T-piece. The vaporiser was initially set at 2% isoflurane.

Postoperatively, the cat received meloxicam (0.2 mg /kg sc, *q* 24 h for 2 days; Maxicam, Sanovel, Turkey), metoclopramide hydrochloride (2 mg/kg iv; Metpamid, Sifar, Turkey) and amoxicillin-clavulanic acid (20 mg/kg sc, *q* 12 h for 5 days; Synulox®, Pfizer, Turkey). An imminent recovery was observed and complete blood count was within normal range on day 3.

The uterus contained a huge number of clear viscous fluid filled sacs with dimensions differing between 0.7-4.5 cm at all six placental sites *(Fig. 1B)*. Estradiol, progesterone, follicle stimulating hormone and testosterone levels in mole fluid were 9.60 pg/mL, 0.04 ng/mL, 0.47 mIU/mL and 0.06 ng/mL, respectively. Embryos of 6-8 mm length were observed at each placental site. Pregnancy age was calculated to be around eighteen days based on the length of the embryos [9]. Conceptus fluids were totally resorbed *(Fig. 2)*. Corpora lutea were observed on both ovaries. Both ovaries contained numerous 1-5 mm in length serous fluid filled cysts. Material for histopathological examination was fixed in 4% buffered formalin, embedded in paraffin and stained routinely with haematoxylin and eosin. Partial villi distention by edema was observed. Affected villi were hydropic and enlarged with irregular scalloped borders. Small nonmolar villi were also evident. Hydropic villi were lined by uniform trophoblastic cells, a few of them showing atypia *(Fig. 3A)*. Some enlarged villi had a central cavity (cistern) containing mucinous matrix. Trophoblastic inclusions in the villous stroma were observed *(Fig. 3B)*. Trophoblastic cells were focally and mildly proliferated and an increased number of endometrial glands, lymphocytes and decidual cells were evident. Vacuolation of the trophoblast cytoplasm and picnotic nuclei were noted focally. Erythrocytes in villous capillaries were observed

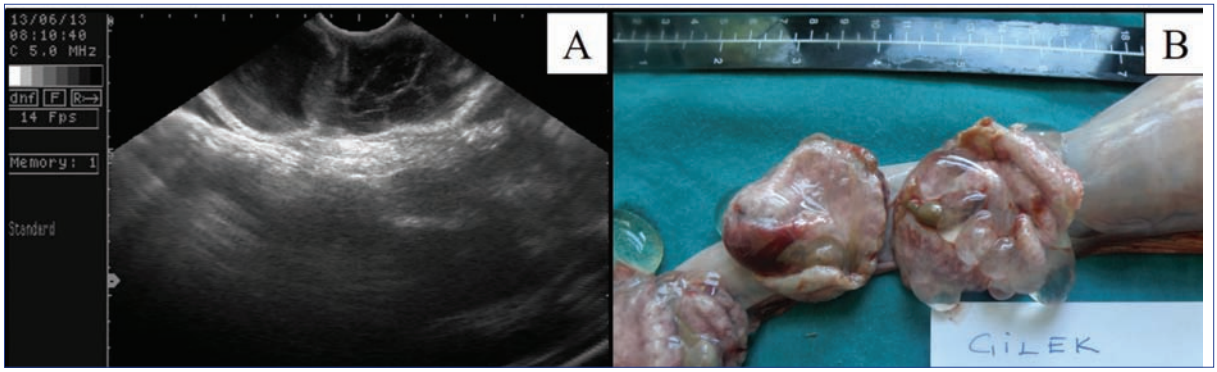

Fig 1. Ultrasonography (A) and macroscopy (B) of partial hydatidiform mole

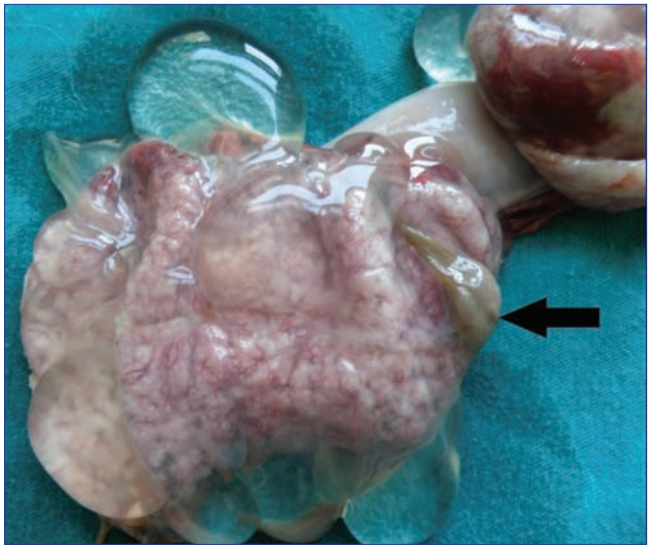

Fig 2. Partial hydatidiform mole-placental changes and embryo *(arrow)*

In addition, trophoblastic inclusions are rarely observed in complete moles, while atypia of trophoblasts are often present [4]. In the current case trophoblastic inclusions were observed frequently and atypia of trophoblasts were less pronounced. Collectively, most of the histopathological features of partial mole in the human were observed in the current case.

Differential diagnosis of hydatidiform mole in the human includes non-molar hydropic abortion with villous edema. In hydropic abortion, villous hydrops is not grossly visible, as it was in our case, rather; it is microscopic and limited. In hydropic abortion, villous shape is round and small; trophoblastic inclusions are usually absent and infrequent small cisterns do not cause gross villous enlargement. Beckwith Wiedemann syndrome or placental angiomatous malformation sometimes mimic partial mole, however, trophoblast proliferation is not observed in these abnormalities [4].

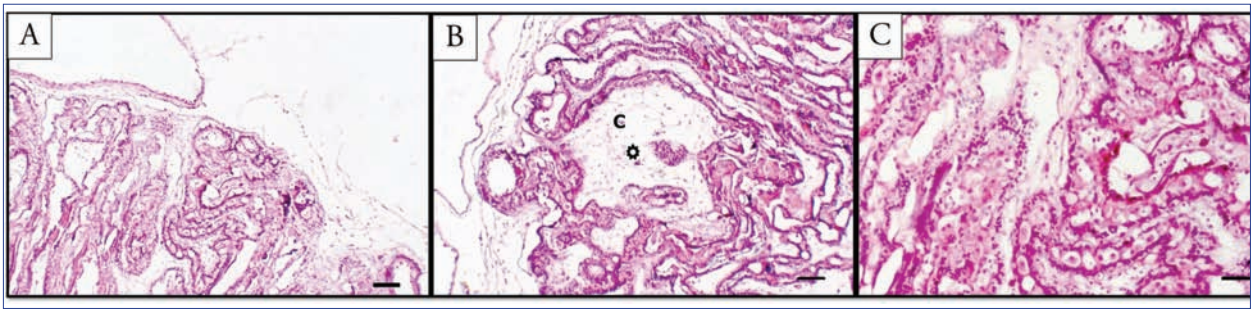

Fig 3. Histopathological findings in partial hydatidiform mole. **A:** Hydropic villi with irregular shape and scalloped borders, bar = 250 µ; **B:** Cystern (C) including mucinous matrix, trophoblastic inclusion *(asteriks)*, bar = 450 µ; **C:** Decidual cells with clear vacuoles, focal lymphocytic infiltrations, villious edema, bar = 300 µ

(Fig. 3C). Based on these histopathological findings and previously reported criteria [4], a diagnosis of a partial hydatidiform mole was made. Histopathology of both ovaries revealed numerous follicular cysts lined by a single layer of cuboidal epithelial cells.

DISCUSSION

Hydatidiform moles which are relatively common in the human and occur in approximately 1 in every 1500 pregnancies. Moles can be complete or partial depending on their gross appearance, histopathology and karyotype [1]. In this case, partial hydatidiform mole diagnosis was made based on the presence of embryos, gross appearance and histopathological findings. Embryo-fetal development usually observed in partial moles is absent in complete moles. In contrast to generalized villi edema with cistern formation and diffuse hyperplasia of trophoblastic tissue in complete moles, villous edema is limited and trophoblastic hyperplasia is focal in partial moles. As a result, some small non-molar villi are also observed in partial moles. These features were clearly evident in the current case.

Our findings are in accordance with gross morphology and histopathology of the only case report in the cat, which describes a stillborn kitten with a triploid karyotype [8]. Authors reported vesicular edematous villi distension, focal mucous stromal degeneration, growth of the trophoblast epithelium and focal cytoplasmic vacuolations of the trophoblastic cells which we also observed. In contrast, atypia of trophoblasts were less pronounced in our case.

Partial moles are usually triploid in karyotype. They contain one maternal and two paternal sets of genes as a consequence of reduplication of the paternal haploid set from a single spermatozoon. In rare cases, partial moles arise from dispermic fertilization of the ovum [10]. In the case with stillborn kitten [8], it was shown that a haploid egg was fertilized by two spermatozoa, resulting in triploid karyotype. Unfortunately, we were not able to perform cytogenetic analysis. However, in our case all six embryos were affected and it seems unlikely that all embryos resulted from dispermic fertilization which is a random event.

Although fluids were totally resorbed, embryos were

not decomposed which shows that embryonic viability was lost recently. In the case with the stillborn kitten, it was reported that some parts of the placenta resembled normal and other parts showed abnormalities. It was also reported that the birth weight of the affected kitten was lower than other newborn kittens of the same breed [8]. In the current case, placentae were highly affected and contained higher numbers of cysts. The severity of the placental lesions might have contributed to different pregnancy outcomes in two cases, with more pronounced placental deficiency and earlier termination of pregnancy in our case. In addition, pregnancy was lost when blood progesterone was above baseline levels, therefore luteal deficiency does not seem to be a contributing factor in termination of pregnancy.

It is interesting to note that uterine enlargement, bloody vaginal discharge, vomiting and blood count abnormalities observed in the current case are among traditional clinical symptoms of hydatidiform mole in the human [10]. The proliferating trophoblastic tissue seems to be the reason for uterine enlargement and vaginal discharge in this case, because uterine interplacental sites were not affected. In early human pregnancy, hCG is primarily produced by the syncytiotrophoblast and excessive amounts of hCG, observed in most cases of hydatidiform mole, leads to medical problems including anemia and hyperemesis gravidarum. In addition, hyperstimulation from increased hCG may cause ovarian enlargement and theca lutein cysts [3]. Although similarities exist between clinical presentations, it should be noted that ovarian cysts we observed were of follicular type and feline trophoblasts do not produce hCG.

In this report, clinical presentation and histopathological findings of a partial hydatidiform mole leading to pregnancy loss in the cat were described.

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Case Report

Unilateral Renal Agenesis in an Aged Dog with Severe Urine Accumulation and Urinary Tract Infection

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Abstract

A 7-year-old intact female Large Münsterländer developed abdominal distention. Computed tomography showed absence of the right kidney and ureter, marked hydronephrosis, and left ureter dilatation. Five years later, the patient was hospitalized as an emergency. Diagnostic imaging showed a >350-mm-diameter cyst communicating with the left kidney displacing the abdominal organs, another approximately 150-mm-diameter cyst in the right kidney position, and marked parenchymal thinning of the left kidney. At laparotomy, a left kidney nephrostomy was established; 8.230 mL of urine were drained with paracentesis. There was no ureter connecting the left kidney and bladder, but an enlarged, ureterocele-like, tubular organ connected the left kidney to a urine-containing cyst on the right side. A short, tubular organ connected the urine-containing cyst on the right side with a bladder-like organ, but it showed almost no urine accumulation. Urine cultures tested positive for Klebsiella pneumoniae. Postoperatively, the patient was discharged after four days. This is the first case about unilateral renal agenesis (URA) in an aged dog. Some dogs may have undiagnosed URA. When URA manifests after a dormant period, cases may be severe, with total loss of appetite, abdominal distension, elevated inflammatory markers, and cyst-like phenomena on abdominal ultrasonography. In such cases, URA must be considered in the differential diagnosis.

Keywords: *Unilateral renal agenesis (URA), Aged dog, Urine accumulation, Urinary tract infection*

Yaşlı Bir Köpekte Şiddetli İdrar Birikmesi ve İdrar Yolu Enfeksiyonu İle Birlikte Unilateral Renal Agenezis

Özet

Yedi yaşında dişi Büyük Münsterländer köpekte abdominal şişkinlik gelişti. Bilgisayarlı tomografide sağ böbrek ve üreter mevcut olmayıp şiddetli hidronefroz ve sol üreterde dilatasyon gözlemlendi. Beş yıl sonra, hasta acilde hastaneye alındı. Tanısal görüntülemede >350mm çapında sol böbrek ile temasta olup abdominal organların yerini değiştiren bir kist ile yaklaşık 150 mm çapında sağ böbrek hizasında bir kist ile sol böbreğin parankiminde belirgin bir incelme tespit edildi. Laparotomide sol böbrek nefrostomisi uygulandı ve 8.230 mL idrar parasentez sırasında drene edildi. Sol böbreği idrar kesesine bağlayan bir üreter bulunmamakla birlikte sol böbreği sağ tarafta idrar içeren bir kiste bağlayan genişlemiş üreterosel benzeri tübüler bir organ gözlemlendi. Sağ tarafta idrar içeren kist ile idrar kesesi benzeri organı bağlayan kısa tübüler bir organ mevcut olup neredeyse hiç idrar toplanması bulunmamaktaydı. İdrar kültürü testi *Klebsiella pneumoniae* için pozitif sonuç verdi. Postoperatif olarak hasta dört gün sonra taburcu edildi. Bu vaka takdimi yaşlı bir köpekte unilateral ranal agenezis (URA) için ilk sunum olma özelliği taşımaktadır. Bazı köpeklerde tanısı konulmamış URA bulunabilir. Dormant dönemi sonrası URA belirince vakalar iştah kaybı, abdominal şişkinlik, artmış yangısal belirteçler ve abdominal ultrasonografide kist benzeri olgu ile birlikte şiddetli seyredebilir. Bu durumlarda URA ayırıcı tanıda düşünülebilir.

Anahtar sözcükler: *Unilateral renal agenezis (URA), Yaşlı köpek, İdrar birikmesi, İdrar yolu enfeksiyonu*

INTRODUCTION

Unilateral renal agenesis (URA) has an incidence of 1 in 500 to 1000 live births in the human population and is a relatively frequent congenital anomaly [1-3] with reportedly no major adverse impact on survival [4]. However, risk factors for URA are proteinuria, renal insufficiency, and hypertension [5], and the relevant risk management should

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be initiated in childhood [6]. URA becomes particularly important in middle and old age [4]. In humans, URA is sometimes associated with uterine and vaginal anomalies [7-9]. The associated menstrual abnormality is reported to result in acute abdominal symptoms [10].

Reports of canine URA date back many years [11,12]. In another study, 50% of dogs with uterine anomalies showed ipsilateral absence of the kidney [13]. URA was also an incidental finding in the case of a 10-month-old bitch undergoing sterilization [14]. Concomitant familial unilateral renal aplasia was found in Dalmatian puppies with recessively inherited acute respiratory distress syndrome [15]. URA was also observed in a three-year-old dog with renal failure [16]. All of these reports involved young dogs: health problems and treatment strategies for older dogs with URA have not been elucidated.

The case of URA in the present report was presented for emergency treatment a long time after the first diagnosis was made. The patient showed marked urine accumulation with impaired renal and urinary function. The patient underwent laparotomy for draining of a giant cyst by paracentesis and establishment of a nephrostomy for the left kidney, and her general condition was alleviated. In this case, we report our observations of a medical emergency that can develop in older dogs with URA.

CASE HISTORY

At the start of this case, the patient, an intact, female Large Münsterländer, was seven years old and weighed 16.5 kg. She was presented for examination at our hospital with abdominal distention as the main complaint (Day 1). She had a normal, healthy appetite and showed no clinical signs other than mild abdominal distension. No specific abnormalities were noted on blood tests (blood urea nitrogen (BUN) 12.0 mg/dL, creatinine (CRE) 0.9 mg/dL). Computed tomography (CT) showed absence of the right kidney and ureter, marked hydronephrosis, and dilatation of the left ureter. With no clinical manifestations other than the mild abdominal distension, a course of follow-up observation by the local veterinarian was decided upon the owner's wish.

Five years after the initial presentation, the 12-year-old patient was hospitalized for reduced appetite, vomiting, diarrhea, and small-scale genital bleeding (Day 1826). She had been in a state of estrus for three weeks previously, and a local veterinarian suspected pyometra after examination with abdominal ultrasound. She was presented at our hospital with a complete loss of appetite and dysstasia (shown over the preceding days) and hospitalized for emergency care. She showed an increased body weight of 23.0 kg, temperature of 39.2°C, heart rate of 64 beats/min, respiratory rate of 20 breaths/min, a pink mucosa, slight dehydration, and marked abdominal distension. Hematology showed a leucocyte count (WBC) of 188×10^2/μL, erythrocyte count (RBC) of 369×10^4/μL, hemoglobin concentration (HGB) of 7.9 g/dL, and a hematocrit value (HCT) of 23.0%. Blood biochemistry revealed a BUN of 43.9 mg/dL, CRE of 1.1 mg/dL, calcium of 10.4 mg/dL, phosphorus of 4.2 mg/dL, elevated C-reactive protein (CRP, inflammatory marker) of 16 mg/dL, and non-regenerative anemia (reticulocytes: 1%; reticulocyte production index: 0.2%). A fluid-retaining, cyst-like lesion, which encompassed most of the abdominal region, was observed by ultrasonography. The patient underwent CT imaging under general anesthesia [induced with subcutaneous atropine at 0.05 mg/kg and intravenous Propofol (Mylan, Mylan Inc., Tokyo, Japan) at 5 mg/kg and maintained with isoflurane inhalation (Isoflurane for animals, Intervet K.K., Tokyo, Japan) at 1.0% to 2.0%]. CT showed a giant cyst (diameter exceeding 350 mm) that involved the left kidney and strongly displaced the abdominal organs into the upper right and left abdominal walls. Marked thinning of the parenchyma of the left kidney was observed. Another cyst (diameter of approximately 150 mm) was located in the position of the right kidney *(Fig. 1)*.

After the CT examination, the patient was moved to

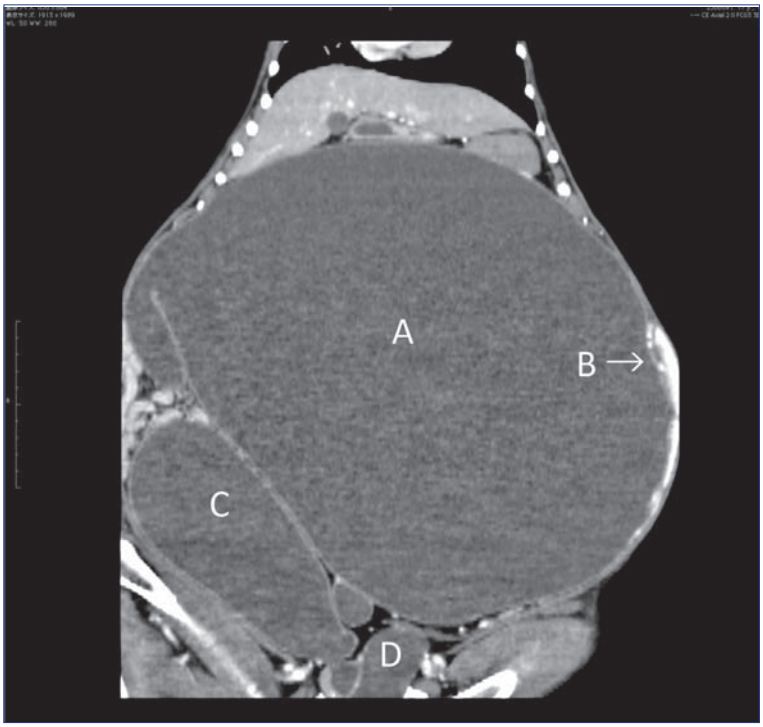

Fig 1. CT examination (Day 1826)
A: the giant cyst (diameter exceeding 350 mm) that involved the left kidney; B: marked thinning of the parenchyma of the left kidney (with contrast effect); C: another cyst (diameter of approximately 150 mm) which was located in the position of the right kidney; D: bladder-like organ

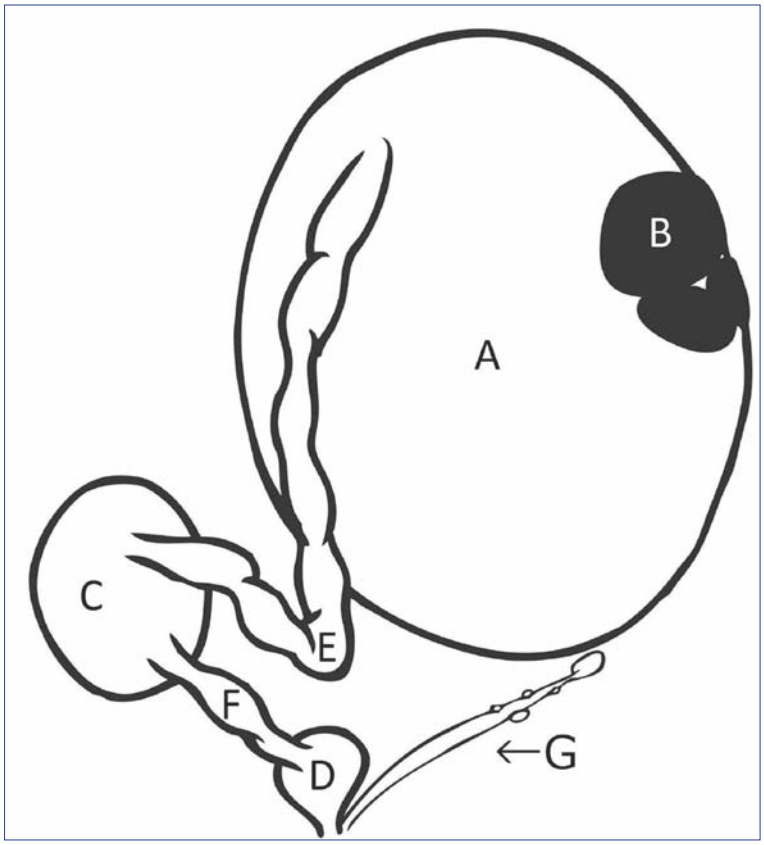

Fig 2. Schema of organs
A: the giant cyst involved the left kidney; B: the parenchyma of the left kidney; C: another urine-containing cyst on the right side; D: bladder-like organ; E: ureterocele-like, tubular organ; F: a short, tubular organ; G: the ovary and uterine horn *(left side)*

the operation room, and the laparotomy was performed. Nephrotomy was conducted on the giant cyst to alleviate the abdominal displacement and to support urination function. The giant cyst involved the left kidney and occupied almost all of the abdominal cavity. 8.230 mL of urine were drained from the cyst. Urinalysis showed a urine-protein-to-creatinine ratio of 1.51. The giant cyst involving the left kidney and another urine-containing cyst on the right side were connected with an enlarged, ureterocele-like, tubular organ (diameter approximately 5 cm), which seemed to derive from the left ureter. The urine-containing cyst on the right side and bladder-like organ were connected with a short, tubular organ; however, the bladder-like organ had no function to expand and showed no urine accumulation. There was no enlargement of the ovary or uterus; however, the ovary and uterine horn ipsilateral to the absent kidney (right side) were also absent *(Fig. 2, Fig. 3)*. A nephrostomy catheter (MILA PEG 20Fr, AVS Co., Ltd., Tokyo, Japan) was placed on the left kidney (20 Fr), and a total ovariohysterectomy was performed.

Urine collected during the surgical procedure was sent for cultivation of bacteria, and it was positive for *Klebsiella pneumoniae*. After surgery, the patient was given fluid (SOLDEM 3, TERUMO Corp., Tokyo, Japan), antibiotic medication (MAXIPIME®, Bristol-Myers Squibb

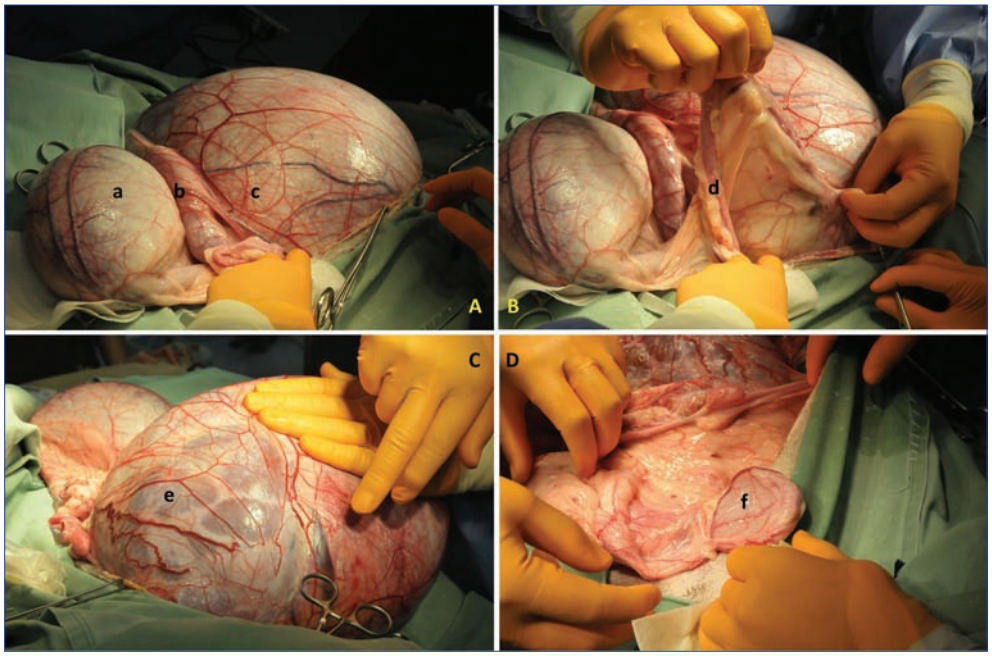

Fig 3. Macroscopic findings
(A) a: another urine-containing cyst on the right side; b: ureterocele-like, tubular organ; c: the giant cyst involved the left kidney; (B) d: the ovary and uterine horn (left side); (C) e: the parenchyma of the left kidney; (D) f: bladder-like organ

Inc., Tokyo, Japan) (cefepime dihydrochloride hydrate, 40 mg/kg, iv, TID), and erythropoietin (Epojin®, Chugai Pharmaceutical Co., Ltd. Tokyo, Japan) (100 IU/kg, s.c., TIW) intravenously. The patient was discharged four days after surgery.

Fifteen days after discharge, the patient weighed 16.7 kg and appeared able to maintain the symptom-free status she had shown when she was seven years old. She is now largely incapable of self-urination and the owner must remove urine from the nephrostomy tube with a syringe twice a day at the home. The daily amount of collected urine is approximately 600 mL (35 mL/kg/day). Her general condition and blood parameters (WBC: 114×10^2/μL, RBC: 490×10^4/μL, HGB: 10.8 g/dL, HCT: 31.4%, BUN: 33.8 mg/dL, CRE: 0.9 mg/dL, Ca: 10.6 mg/dL, P: 4.8 mg/dL, CRP: 1.95 mg/dL) have shown great improvement.

DISCUSSION

The URA patient in the present study was able to lead an active life up to the advanced age of 12 years without receiving any special medical treatment and without showing clinical manifestations. Because URA is frequently reported in people as an incidental finding and has no major adverse impact on survival [4], a similar situation may occur in dogs. In the same way that human URA patients can develop a condition requiring emergency care, old canine URA patients can take a sudden turn for the worse. However, the cause of the sudden exacerbation in this case seemed to be different from those in human medicine, which are related to reproductive organ disorders [10]. The sudden exacerbation in this canine case may have resulted from the following factors: abdominal organ displacement by a giant renal cyst within the abdominal cavity or an opportunistic urinary tract infection (UTI) under impaired renal and urinary function. Therefore, the therapeutic strategy focused on alleviating the abdominal organ displacement and palliating the general condition quickly. For this purpose, nephrostomy-assisted urination and a course of antibiotics to manage the UTI was conducted. URA sometimes occurs with ipsilateral absence of the ureter and a normal, contralateral kidney and ureter [14], with contralateral renal dysplasia [16], or with a normal, ipsilateral ureter [12]. The findings in the present case showed not only the absence of the right kidney and anomalies in the left kidney, but also a specific pattern of urinary transit in which the left ureter was connected to the urine-containing cyst on the right side rather than to the bladder. The very large amount of urine accumulation has not been reported in the previous reports and appears to be a characteristic finding in the present case. Because an increased CRE was not seen, it is possible that the elevated BUN arose mainly from the hypermetabolism related to dehydration and loss of appetite on Day 1826. It is possible that the increased BUN originated with the accompanying improvement of the physical status, because the patient took a predominantly meat diet on Day 1845.

Deficient elimination of residual urine from the bladder is often a factor implicated in UTI onset; however, in this case, the bladder failed to expand and there was very little urine accumulation. It appears that urine accumulation in the kidney and ureter were a regular event for this patient. Vesicoureteral reflux is reported in human URA [3]. The presence of vesicoureteral reflux could not be determined in this case, but the markedly decreased urine accumulation in the non-expanding bladder was likely associated with the accumulation of large quantities of urine in the upper urinary organs. Although the urine accumulation in the left kidney appeared to reach a severe level, the patient did not show any clinical signs for a long time. CT findings showed very little fluid accumulation in the bladder-like organ, and most of the fluid had accumulated in the cyst-like structure over a long period of time. *Klebsiella pneumoniae*, which, is sometimes reported as a canine UTI pathogen, was detected by urinary cultures [17]. It seems that this aggravation of clinical status was induced by multiple factors including UTI, which were caused by the urine accumulation. In addition to the UTI, urine that accumulated within the giant cyst also caused severe displacement of the abdominal organs, causing the general condition of the patient to be exacerbated.

Although URA is a relatively common congenital anomaly in humans, it has been reported only sporadically in comparatively young dogs. Accordingly, the details of canine URA are not clearly understood. The dog in this case reached an advanced age without showing clinical symptoms, and it seems that a not inconsiderable number of dogs exist with undiagnosed URA. When URA manifests after a period of clinical silence, patients may show total loss of appetite, abdominal distension, elevated inflammatory markers, and abdominal ultrasonography images of cyst-like phenomena. The findings are similar to pyometra; however, URA should be considered in the differential diagnosis.

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Review

Geriatric Cardiology in Dogs - Part 1: Classification and Treatment of Heart Failure in Geriatrics

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Abstract

The purpose of this article is to give current information on the effect of aging on heart, clasification of heart failure (HF) in geriatric dogs and therapeutic advances and challenges. The aging process results in a host of physiological and biological changes that lead to progressive HF. Nearly any cardiovascular disease (CVD) can lead to HF. HF in geriatric dogs is broadly classified into: Diastolic heart failure is defined as HF with preserved ejection fraction (HF/PEF), systolic heart failure is also known as HF with low ejection fraction (HF/low-EF). Importantly, HF/PEF accounts for approximately 50% of all HF patients and its prevalence is higher in the elderly. In humans, mortality and morbidity benefits for HF/low-EF have been reported with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs), β-blockers and aldosterone antagonists, and the hydralazine–nitrate combination in patients intolerant to ACE inhibitors or nitrates. Therapies for symptom control and morbidity benefit include diuretics and digoxin. The most common and suggested therapy for dogs with HF by ACVIM panelists is furosemide, ACE-I and pimobendan. Pharmacological therapy is limited for HF/PEF as the results of small trials have been inconclusive. To date, clinical trials in HF/PEF patients have not shown mortality benefit so that management is focused on symptom relief and treatment of the underlying cause.

Keywords: *Geriatric Cardiology, Classification, Treatment, Heart Failure, Dog*

Köpeklerde Geriatrik Kardiyoloji - Bölüm 1: Geriatriklerde Kalp Yetmezliğinin Klasifikasyonu ve Tedavisi

Özet

Bu derlemede yaşlanmanın kalp üzerine etkileri, geriatrik köpeklerde kalp yetmezliğinin (HF) klasifikasyonu ve terapötik yaklaşımları konularında güncel bilgiler vermek amaçlanmıştır. Yaşlanma progressif HF'ne yol açan fizyolojik ve biyolojik değişikliklere yol açar. Hemen hemen tüm kardiyovasküler hastalıklar (CVDs) HF'ne yol açabilir. Geriatrik köpeklerde, HF başlıca; diyastolik kalp yetmezliği, HF ile korunmuş ejeksiyon fraksiyonu (HF/PEF) olarak, sistolik kalp yetmezliği, HF ile düşük ejeksiyon fraksiyonu (HF/düşük-EF) olarak klasifiye edilir. Tüm HF hastalarının ortalama %50'sini HF/PEF oluşturur ve geriatriklerde prevalansı daha yüksektir. Geriatrik insanlarda HF/düşük EF için tedavi başlıca ACE inhibitorleri veya ARBs, β-blokörler, aldosterone antagonistleri ve ACE inhibitorleri veya nitrat intöleransı olan hastalara hydralazine–nitrat kombinasyonunu kapsar. Semptomların kontrolu ve morbidite faydası için diüretikler ve digoxin kullanılır. Kalp yetmezliği olan köpeklerde ACVIM panelislerince tavsiye edilen tedaviyi furosemide, ACE-I, ve pimobendan oluşturmaktadır. HF/PEF olan hastalardaki klinik çalışma verileri mortalite faydası olmadığını göstermiştir. Tedavi semptomların iyileştirilmesi ve primer nedenin tedavisine yönelik yapılır.

Anahtar sözcükler: *Geriatrik kardiyoloji, Klasifikasyon, Tedavi, Kalp yetmezliği, Köpek*

INTRODUCTION

Why geriatric cardiology is important?
Is the term geriatric cardiology redundant?

Technically, geriatric cardiology refers to cardiovascular care of dogs 9 years of age or older (giant breed >7.5 years) [1]. It is the practice of cardiovascular (CV) medicine that is adapted to the needs of older dogs. To some degree, all cardiologists know this, recognize this, and in varying capacities, practice this.

Not surprisingly, a recent survey in Near East University, Veterinary Hospital found that almost 50% of patients who

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visited Hospital over the last year would be considered geriatric on the basis of age alone.

But, is there reason to believe that cardiovascular management of these older dogs differs from that of younger cardiovascular patients?

A clue comes from the mission statement of the geriatric cardiology member section of the American College of Cardiology (ACC), which states that the practice of geriatric cardiology should consider "*all matters related to cardiovascular care-giving in relation to aging*"[2].

In older dogs, multiple medical problems (hypertention, diabetes, hyperlipidemia, frailty, cognitive dysfunction (CD) lead to polypharmacy. Compounding these burdens is that older patients have not only considerable clinical needs, but psychological and social needs too [3-5].

In humans, polypharmacy, defined as the concurrent use of 5 or more medications, occurs in up to 40% of older patients. Absorption, bioavailability, and volume of distribution of pharmacologic agents change dramatically with age, significantly increasing the risk of drug interactions, particularly in patients with polypharmacy [6]. Importantly, the risk of adverse drug effects is about 50% in patients on 4 chronic medications and approaches 100% in those taking more than 7. Moreover, almost one-half of hospitalizations related to adverse drug events are attributabe to cardiovascular medications (particularly diuretics, warfarin, beta-blockers, and angiotensin-converting enzyme inhibitors) [7]. The challenge is to prioritize treatments, minimize dangerous interactions, and optimize quality of life [3].

Recommendations require thoughtful individualization, based on integrating patient-centered priorities with respect to each patient's aging experience. This article focuses on the effect of ageing on heart, clasification of HF in geriatric dogs and therapeutic advances and challenges.

EFFECTS OF AGING ON THE CARDIOVASCULAR SYSTEM

An accurate biomarker of aging is lacking. Pathophysiologic hallmarks of cardiovascular aging are summarized in *Table 1* [8-10].

THE IMPORTANT CARDIOVASCULAR DISEASES IN GERIATRIC DOGS

Most dogs born with congenital heart disease died when they were young and did not reach old age or had the defect corrected surgically (eg, patent ductus arteriosus), and the defect is thus of no concern in old age.

The aging process results in a host of physiological and biological changes that lead to progressive HF *(Table 1)*. Nearly any cardiovascular disease (CVD) can lead to HF. Common CVDs causing HF in geriatric dogs are given in *Table 2* [1,4,5,11,12].

HEART FAILURE

Heart failure (HF) is a major healthcare burden in dogs and a particularly important problem in the elderly dogs. This is the most common disorder in small animals' cardiorespiratory clinics, commonly the end-stage in a CVD continuum *(Fig. 1)*. It is characterized with progressive decrease in cardiac output and ventricular filling [5,13].

Definition of HF: Definitions of HF in dogs and humans is based on a clinical syndrome that emphasizes symptoms and signs [5,11]. Briefly, the HF syndrome consists of a triad of;

Typical symptoms; exercise intolerance, weakness, weight loss, chronic cough, peripheral edema, orthopnoea, syncope, inappetence.

Typical signs; tachyarythmia, tachypnea, pulmonary rales, ascites, pleural effusion, raised jugular venous pressure, hepatomegaly.

Objective evidence of structural and functional abnormality; cardiomegaly, cardiac murmurs, abnormal echocardiogram or raised atrial natriuretic peptide marker level.

Pathophysiology: Most HF patients have myocardial dysfunction. Common causes include muscle damage or loss from mitral regurgitation, hypertensive (pulmonary/systemic) cardiomyopathy, atherosclerosis, diabetic cardiomyopathy, increased vascular resistance and afterload from hypertension, and increased heart rate from tachyarrhythmias such as atrial fibrillation [4,5,11,14].

Early cardiocirculatory, hemodynamic and cardio-renal HF models emphasized decreased pump function, cardiac output and renal blood flow, and increased peripheral vasoconstriction and provided the rationale for using diuretics, inotropes and vasodilators [15].

Subsequently, the neurohumoral model emphasized the development and progression of asymptomatic to symptomatic HF. In this construct, injury leads to activation of the adrenergic nervous system (ANS), the renin-angiotensin-aldosterone system (RAAS) and cytokine systems that preserve LV function in the short term, and to secondary damage with maladaptive LV remodeling and worsening HF in the long term. After that, LV remodeling is severe and impaired healing can further aggravate remodeling in the elderly [15,16].

Whereas activation of the ANS decreases during the progression of HF resulting in decreased norepinephrine (owing to an exhaustion phenomenon) and reuptake, RAAS activation persists. The neurohumoral model provided

Table 1. *Pathophysiological changes and pathophysiologic hallmarks during aging*
*dysregulation of repair mechanisms: decreased endothelial nitric oxide production and increased endothelial apoptosis, superoxide production and advanced glycation end product
*fibrotic cardiac remodeling: diastolic and systolic dysfunction
*remodeling of the vascular wall: arterial stiffening
*increased collagen, decreased elastin and calcification: atrial fibrillation, bradyarrhythmias
*increase in fibrinogen, coagulation factors, platelet activity, plasminogen activator inhibitor-1, prothrombotic cytokines: atherosclerosis
*enhanced adherence of mononuclear cells to the endothelial surface, promoting an inflammatory microvascular environment: tromboembolizm
*abnormal adrenergic responses: blunted β1-adrenergic myocardial contractility and β2-adrenergic vasodilatation
*limited ATP production: less contraction or relaxation in response to stress

Table 2. *The most common diseases causing HF in geriatric dogs*
*mitral regurgitation; valvular diseases, DCM
*hypertensive (pulmonary/systemic) cardiomyopathy
*atherosclerosis
*diabetic cardiomyopathy (hyperlipidemia, Type 2 diabetes)

aldosterone secretion and catecholamine release, whereas AT_2 activation leads to vasodilation, the inhibition of cell growth, natriuresis and bradykinin release. The ratio of AT_1 to AT_2 receptors decreases in HF. Sustained expression of angiotensin II leads to cardiac and renal fibrosis (partly through increased aldosterone), cardiac hypertrophy, and maladaptive cardiac and vascular remodeling. Aldosterone

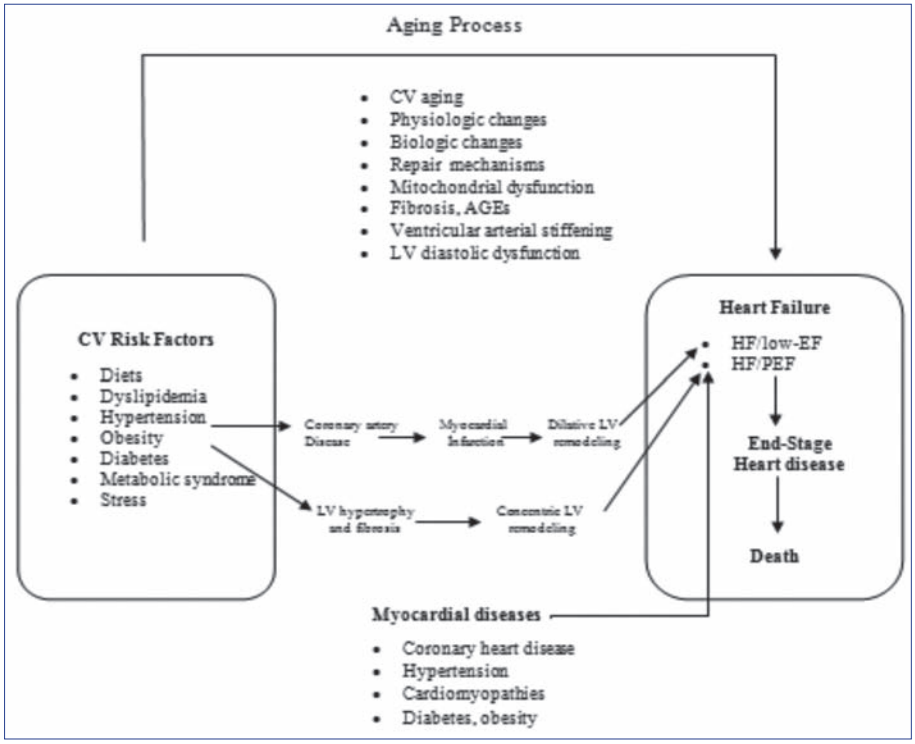

Fig 1. Heart failure, aging and the cardiovascular disease continuum. Heart failure can be considered as a progressive disorder in a disease continuum occurring in parallel with aging and leading to disability and ultimate death. AGE: Advanced glycation end product; CV: Cardiovascular; EF: Ejection fraction; HF: Heart failure; LV: Left ventricular; PEF: Preserved ejection fraction

rationale for using ACE inhibitors, ARBs, aldosterone antagonists and β-blockers in HF [15].

While most angiotensin II, the major effector molecule of the RAAS, is formed via the ACE-dependent pathway, angiotensin II can also be produced through renin- and ACE-independent pathways. Angiotensin II acts through the angiotensin II type 1 (AT_1) and angiotensin II type 2 (AT_2) receptors. The predominant subtype is AT_1 in the vasculature and AT_2 in the myocardium, with localization of AT_1 in nerves and AT_2 in fibroblasts and the interstitium. AT_1 activation leads to vasoconstriction, cell growth, also leads to the inhibition of norepinephrine re-uptake and worsening HF [4,5,15,17].

Clasification of HF: The new ACVIM (American College of Veterinary Internal Medicine) classification of cardiac disease that was adapted from the American College of Cardiology and uses an A-through-D categorization scheme (stage A with risk of HF but no structural changes or symptoms; stage B with structural changes but no signs or symptoms; stage C with structural changes and prior or current symptoms; stage D with severe refractory HF requiring specialized interventions [5,18,19].

Currently, HF in geriatric dogs is considered to be the result of structural and functional cardiac disorders that impair ventricular filling and ejection, and is broadly classified into:

Diastolic heart failure (DHF), HF with preserved ejection fraction (HF/PEF).

Systolic heart failure (SHF), HF with low ejection fraction (HF/low-EF).

Importantly, HF/PEF accounts for approximately 50% of all HF patients and its prevalence is higher in the elderly [20,21].

Treatment of HF: Although HF is primarily a disorder of the elderly, there is a lack of evidence-based data from randomized clinical trials (RCTs) of HF, specifically in elderly patients and geriatric dogs. More often drugs have to be chosen based on pathophysiological logic. Dose adjustments are necessary so as not to cause harm [4,5].

Therapies for HF/low-EF: In humans, mortality and morbidity benefits have been reported with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs), β-blockers and aldosterone antagonists, and the hydralazine–nitrate combination in patients intolerant to ACE inhibitors or nitrates. Therapies for symptom control and morbidity benefit include diuretics and digoxin [7,18].

The most common and suggested therapy for dogs with HF by ACVIM panelists is furosemide, ACE-I, pimobendan. For the following drugs, no consensus has been reached but many cardiologists use spironolactone for its diuretic, potassium-sparing and presumably anti-fibrotic properties, digoxin for rate control, beta adrenergic blockers in cases of atrial fibrillation to control the ventricular rate, or ventricular arrhythmias. Other drugs that can be utilized and are used by some veterinarians are amlodipine (up to 0.1 mg/kg q12 h, needs to be titrated and monitor blood pressure), hydrochlorthiazide (2-4 mg/kg q12 h) and torsemide (0.2 mg/kg q12-24 h) instead of furosemide. These last three drugs are usually reserved for refractory heart failure cases [18,22].

Class D1 and D2 dogs have clinical signs of congestive and or/low output heart failure. Usually they need pleural or abdominal centesis, oxygen, nursing care. Other drugs can be added, such as cough suppressants, bronchodilators, sildenafil, but no consensus has been reached on them [18,22].

- ACE Inhibitors: ACE-Is are the first choice in HF pharmacotherapy. They are recommended in patients with HF/low-EF (i.e., EF <40%) regardless of the presence of HF symptoms based on the RCTs [23-26].

A review of 34 RCTs was published and showed decreased mortality and hospitalization. The benefit was primarily related to fewer deaths from progressive HF and was found with enalapril, captopril, ramipril, quinapril and lisinopril, suggesting a class effect [27]. However, ACE-I did not meet consensus by the group at C1 stage of the disease. ACE-I (dose depends of the drug used) (Enalapril 0.5 mg/kg, 1-2x/d p.o.; Benazepril 0.25-0.5 mg/kg, 1-2x/d p.o.) can be given Class C2 and D dogs [5,18,22].

Although every attempt should be made to use target doses shown to produce mortality benefit in RCTs, it is advisable in elderly patients to start at a low dose and uptitrate to the tolerated dose. Similar precautions are needed in elderly HF patients [4,11]. Over 75% of patients still do not receive the optimal dose of ACE inhibitors. In elderly patients who cannot tolerate the target dose, a lower dose of an ACE inhibitor is still beneficial but a β-blocker should be introduced [11].

- Angiotensin Receptor Blockers: Most RCTs for ARBs used ACE inhibitors as comparator and patients received other background therapy such as β-blockers and/or diuretics. In the Valsartan Heart Failure Trial (Val-HeFT), overall mortality was similar in valsartan and placebo groups but combined morbidity and mortality was lower with valsartan [4,28].

In contrast to Val-HeFT, the candesartan in Heart Failure: Assessment of Reduction in Mortality and Morbidity (CHARM)-Added trial with candesartan on top of an ACE inhibitor and background therapy showed a reduction in the primary end point (hospitalization or death) [29]. In the CHARM- Alternative trial, use of candesartan in ACE inhibitor-intolerant patients reduced morbidity and mortality [30]. However, 7.7% of the patients developed angioedema.

Similar precautions as with ACE inhibitors should be observed with ARBs [11]. It is reasonable to add a β -blocker before the target dose of an ARB is reached in stable patients.

- Pimobendan: Pimobendan does increase contractility without increasing myocardial oxygen consumption. In addition, pimobendan exerts a peripheral vasodilatatory effect by phosphodiesterase III inhibition, which may not only be important in the systemic but also in the pulmonary circulation. Furthermore, the vasodilation affects arteries as well as veins, therefore it is expected to reduce afterload and preload. Finally, it may have an anticytokine effect. The ACVIM panelists suggest also using pimobendan at 0.1-0.3 mg/kg q 12 h in dogs with HF [18,22]. It can be combined with many drugs, e.g. furosemide, ACE inhibitors, betablockers, digoxin and amlodipine [5].

β-blockers. The majority of RCTs added a β-blocker to background therapy that included ACE inhibitors. The addition of a β-blocker to an ACE inhibitor and a diuretic is recommended in clinically stable stage C patients with or without symptoms [11]. Compelling evidence of mortality benefits exists for bisoprolol, sustained-release metoprolol succinate and carvedilol. A meta-analysis of five RCTs with more than 12.000 patients, of whom 4617 (36.3%) were elderly, showed mortality benefits with those three

β-blockers [4,31,32]. β-blockers are therefore recommended in elderly patients with HF/low-EF.

In dogs with HF, β-blockers can be used in cases of atrial fibrillation to control the ventricular rate, or ventricular arrhythmias [18].

Mitochondria in hearts of aged patients seem to be unable to produce increased amounts of ATP to fuel contraction or relaxation in response to stress. The β-adrenergic blocking agents may improve exercise capacity dramatically, in human.

Which of these issues is applicable to the aging pet is unknown [8].

Initiation with the lowest tolerated dose and gradual escalation reduce the risk of hypotension and bradycardia. When hypotension and bradycardia are accompanied by evidence of hypoperfusion (i.e., cold extremities, or worsening of renal or hepatic function), the dose should be decreased or the drug withdrawn (Carvedilol, 0.05-0.4 mg/kg, 1-2x/d p.o.; Atenolol, 0.2-1.5 mg/kg, 1-2x/d p.o.) [4,5].

- *Diuretics:* Patients with a history or symptoms of HF and evidence of fluid retention should be treated with sodium restriction and diuretics such as loop diuretics (furosemide, torsemide and bumetanide), thiazides (metolazone and hydrochlorthiazide) or aldosterone antagonists (spironolactone and eplerenone). Loop diuretics increase sodium excretion (by 25% of filtered load) and free-water clearance unless renal function is severely impaired (creatinine clearance <10 mL/L). Thiazides increase fractional sodium excretion (by 5-10% of filtered load) and decrease free-water clearance unless renal function is moderately impaired (creatinine clearance <30 mL/L) [4,5,33-35].

The ACVIM panel suggested for C1 dogs, furosemide 1-4 mg/kg IV, IM or SC as bolus or 1 mg/kg, constant rate infusion (CRI), the latest being reserved for dogs not responding to a bolus injection. In some cases higher doses of furosemide such as 4-8 mg/kg can be given in severe pulmonary edema cases; the dose depends on the kidney status of the animal, the amount of water taken and age. Monitoring respiratory rate, ECG, urine output and drinking is essential [5,18,33].

Furosemide (1-2 mg/kg q12 h to 4-6 mg/kg q 8 h orally) can be given to Class C2 and D1, D2 dogs with careful monitoring of renal parameters; mild azotemia is unavoidable and well tolerated. Diuresis can be increased by higher dosage or increased frequency of application, also in refractory cases furosemide can be given by SQ injection by the owner instead of a tablet. In these cases, additional diuretics are added like hydrochlorthiazide (1-2 mg q 12-24 h), spironolactone (0.5-2 mg/kg q 24 h) or torsemide (0.1 × dose of furosemide) [5,18,33].

Hydrochlorthiazide (2-4 mg/kg q 12 h) and torsemide (0.2 mg/kg q 12-24 h) instead of furosemide can be used in C2 dogs [5,18,33].

Patients on diuretics should be closely monitored. The dose should be individualized, especially in the elderly who are susceptible to orthostatic hypotension and renal dysfunction from over-diuresis. Too low a dose can lead to fluid retention, which in turn can blunt the response to ACE inhibitors and decrease tolerance of β-blockers. Too high a dose can lead hypovolemia, increased susceptibility to hypotension from ACE inhibitors, ARBs and/or other vasodilators, and renal insufficiency from ACE inhibitors or ARBs [4].

In the elderly, it is prudent to initiate therapy with a low dose and titrate upward based on the diuretic response and weight loss. Hypokalemia and hypomagnesemia should be promptly treated to prevent arrhythmias, especially if digoxin is being used. When a loop diuretic is used, an ACE inhibitor or ARB alone, or in combination with an aldosterone antagonist, may reduce the risk of electrolyte depletion [5,18,33].

- *Aldosterone Antagonists*: An important aspect of the RAAS is the AT_1 receptor-mediated activation of aldosterone, which promotes sodium retention, loss of magnesium and potassium, sympathetic activation, parasympathetic inhibition, myocardial and vascular fibrosis, baroreceptor dysfunction, vascular damage and impaired arterial compliance. The rationale for using aldosterone antagonists is that angiotensin II stimulates the release of aldosterone, thereby activating the mineralocorticoid receptor, and the activation of this receptor persists despite the use of ACE inhibitors, ARBs and β-blockers [5,36,37].

While aldosterone antagonists are typically used for their mortality benefits, long-term studies of other diuretics on morbidity and mortality in HF are lacking [36,37]. In dogs with HF, spironolactone is used for its diuretic, potassium-sparing and presumably anti-fibrotic properties [5,18,33].

Based on evidence from two RCTs [34], the addition of an aldosterone antagonist is considered reasonable in patients with moderate-to-severe HF and reduced EF, provided renal function (serum creatinine ≤2.5 mg/dL in men and ≤2.0 mg/dL in women) and serum potassium ≤5.0 mEq/L) can be carefully monitored [11].

The Randomized Aldactone Evaluation Study for Congestive Heart Failure (RALES), which assessed spironolactone on top of background therapy with ACE inhibitors, β-blockers, diuretics and digoxin in patients with moderate-to-severe HF (EF <35%), was prematurely terminated owing to an early finding of a 30% reduction in all-cause mortality but also reduced morbidity and hospitalization [34].

- *Digoxin:* The addition of digoxin is recommended if HF symptoms persist despite optimal treatment with an ACE inhibitor, β-blocker and/or a diuretic [11]. The Randomized

Assessment of Digoxin on Inhibitors of Angiotensin-Converting Enzyme (RADIANCE) study reported worsening after the withdrawal of digoxin in HF patients who were in sinus rhythm and stable on a regimen of digoxin, diuretics and ACE inhibitors [38].

In dogs with HF, digoxin (0.22 mg/m^2) can be used for rate control [18]. Since digoxin has a narrow therapeutic index and is excreted by the kidneys and the elderly are at risk of renal dysfunction and/or lean body mass loss, a low initial dose of 0.125 mg daily or every other day should be used in patients aged over 70 years [11]. While the serum digoxin level does not seem to correlate with clinical benefits, a serum digoxin level over 1 ng/mL is associated with increased mortality, in humans [39]. A level less than 1.2 ng/mL might therefore be safer for dogs [5].

- *Vasodilators*: Based on three RCTs, the addition of a combination of isosorbide dinitrate and hydralazine is considered reasonable if the symptoms persist despite ACE inhibitor and β-blocker therapy, or if patients are intolerant to ACE inhibitors or ARBs [11]. Val-HeFT I reported that the addition of isosorbide dinitrate and hydralazine can be favourable in male patients with mild-to-severe HF (EF <45%) receiving digoxin and diuretics (Isosorbide dinitrate ½-1 hub, rubben on hairless skin; Hydralazine 0.5-3 mg/kg orally q 12 h for dogs) [4,40].

Amlodipine (0.15-0.25 mg/kg SID, needs to be titrated and monitor blood pressure) can be used in C2 and D1 dogs, especially in cases with high blood pressure because of renal failure [5,18].

Medical Therapy of HF/PEF: Heart failure/PEF increases with age, is very common in the elderly [21]. It considers HF/PEF as a triad of [41]: (1) signs and symptoms of HF, (2) normal or mildly abnormal LV systolic function (LV EF >50% or LV end-diastolic volume index <97 mL/m^2), and (3) evidence of LV diastolic dysfunction (echocardiographic/Doppler E/E' >15, E'< 8 cm/sn or elevated NTproBNP concentration >450 pmol/L with abnormal echocardiographic/ Doppler parameters) [5,42].

In contrast to HF/low-EF, patients with HF/PEF are more likely to be elderly, and hypertensive, and less likely to have previous therapy with ACE inhibitors and ARBs [43].

Mortality is similar to that with HF/low-EF. While survival over time has improved for HF/low-EF, it has remained unchanged for HF/PEF. Major causes of HF/PEF in the elderly include LV hypertrophy, hypertrophic cardiomyopathy, aortic stenosis with normal EF, ischemic heart disease, restrictive cardiomyopathy with idiopathic causes or infiltrative cardiomyopathy [44]. In humans, it is believed that systolic dysfunction is a consequence of diastolic dysfunction because of decreased preload [4].

The nonpharmacological strategy is similar to that for patients with HF/low-EF (i.e., daily monitoring of weight, attention to diet and lifestyle, and close medical follow-up) together with aggressive control of hypertension, tachycardia and other precipitating causes of de-compensation. Emerging evidence suggests that exercise training improves outcome (i.e., improves quality of life, increases longevity and decreases symptoms) [4].

Pharmacological therapy is limited as the results of small trials have been inconclusive. To date, clinical trials in HF/PEF patients have not shown mortality benefit so that management is focused on symptom relief and treatment of the underlying cause. Beyond, the established beneficial effects of ACE inhibitors in patients with hypertension that may be due, at least in part, to attenuation of cardiac fibrosis or other anti-fibrotic strategies (such as AGE breakers) may exert beneficial actions in high-risk elderly patients with diastolic heart failure [9]. In the Perindopril in Elderly People with Chronic Heart Failure (PEP-CHF) trial, the ACE inhibitor perindopril in patients aged over 70 years (mean: 76 years of age) and HF-PEF did not reduce the primary end point but the event rates were lower than anticipated [26]. However, there was a trend towards reduced HF hospitalization at 1 year.

In the CHARM-Preserved trial in patients with mild-to-moderate HF and LV EF more than 40%, the ARB candesartan on top of standard therapy had no mortality benefit; 22% of patients in the candesartan group and 24% in the placebo group reached the primary end point (cardiovascular death or HF hospitalization) [45].

In the irbesartan in Patients with Heart Failure and Preserved Ejection Fraction Study (I-PRESERVE), the ARB irbesartan in 4128 patients aged 60 years or over (mean: 72 years of age) showed no benefit [46].

In a substudy of the Digitalis Investigation Group (DIG) trial, digoxin did not change the primary end point of HF hospitalization or cardiovascular mortality [47].

In summary, specific medical therapy for HF/PEF is lacking. Current recommendations for the treatment of HF/PEF include [4,11]: (a) control of systolic and diastolic hypertension, (b) control of ventricular rate in patients with atrial fibrillation, (c) control of pulmonary congestion and peripheral edema with diuretics, (d) treatment of coronary artery disease and demonstrable myocardial ischemia with coronary revascularization, (e) restoration and maintenance of sinus rhythm in patients with atrial fibrillation, (f) use of digoxin in selected patients.

Other HF Treatments

- *Supraventricular Arrhythmias:* Both patients with HF/PEF and HF/low-EF develop atrial fibrillation and other supraventricular arrhythmias [48]. Deleterious effects of supraventricular tachyarrhythmias are due to: (1) loss of atrial contribution to ventricular filling, (2) increased myocardial oxygen demands and decreased perfusion, (3)

impaired contraction and relaxation, (4) stasis of blood in the atria leading to thrombus formation and embolism, (5) the development of cardiomyopathy from sustained tachycardia [11].

Atrial fibrillation is the most common treatable supraventricular or atrial arrhythmia. It leads to decreased exercise capacity and aggravates long-term prognosis. Atrial fibrillation begets atrial fibrillation, which begets HF. It is also more common in the elderly [49].

Two important aspects of therapy in HF patients are rate and/or rhythm control and prevention of thromboembolism. The goal is to decrease the ventricular rate to less than 80-90 bpm at rest and to less than 110-130 bpm with moderate exercise [11].

Digoxin is more effective in slowing atrioventricular

(AV) conduction at rest than during exercise. β-blockers are more effective than digoxin during exercise. The combination of digoxin and β-blockers may be more effective for rate control. When β-blockers fail to control HF or are contraindicated, amiodarone (10 mg/kg orally q 12 h for 7-14 days or 10-15 mg/kg orally q 24 h for 7-14 days, than 5-7.5 mg/kg orally q 24 h for dogs) can be used. If pharmacotherapy fails to control heart rate, AV-node ablation is indicated. R-wave synchronized cardioversion is recommended when rapid rate is unresponsive to therapy for patients with ischemia, hypotension angina or HF [48].

Anticoagulation should be maintained in all patients with atrial fibrillation regardless of whether sinus rhythm is restored owing to the high recurrence rate with associated embolic risk [5].

- Ventricular Arrhythmias, Sudden Death: Both patients with HF/PEF and HF/low-EF can develop ventricular arrhythmias and sudden death. Sudden death can result from two main causes: (1) arrhythmias, usually ventricular tachyarrhythmias such as non-sustained or sustained ventricular tachycardia (VT) but less often bradycardia and pulseless supraventricular rhythms, especially in stage D HF, (2) ischemia with ACS and acute MI, electrolyte imbalance and vascular events, including pulmonary and systemic embolism [11].

In addition, HF/low-EF patients with dilative LV remodeling are prone to develop VT (non-sustained or sustained) and sudden death besides non-sudden death from pump failure [11].

Definitive therapy for myocardial ischemia or other reversible factors can prevent the recurrence of tachyarrhythmia such as polymorphic VT, non-sustained VT and ventricular fibrillation. Medical therapies that decrease disease progression such as Lidocain (2 mg/kg i.v. over 1-2 min (repeat in 2 mg/kg boluses up to 8 mg/kg in 10 min) or 0.8 mg/kg/min i.v. infusion (up to 8 mg/kg)), β-blockers [32,50] and aldosterone antagonists [36,37] can decrease sudden deaths in HF/low-EF patients.

In addition, the negative inotropic effects of antiarrhythmic drugs is a concern as they may aggravate HF and augment the risk of serious arrhythmias in HF/low-EF patients [5].

Importantly, in humans, implantable cardioverter-defibrillator (ICD) devices that terminate sustained arrhythmias can further decrease sudden death in that group. ICDs are therefore recommended for all patients with life-threatening tachyarrhythmias and otherwise good prognosis [11].

Patients with stage D HF/low-EF often (in 33%) have a QRS duration of more than 0.12 s and LV dyssynchrony or delayed activation of the LV free wall. Ventricular dyssynchrony is associated with increased mortality in HF patients. Previously known as biventricular pacing, cardiac resynchronization therapy (CRT) re-coordinates ventricular beating by the insertion of atrial-synchronized biventricular pacing catheters, thereby eliminating dyssynchrony [51]. CRT can also reduce secondary mitral regurgitation.

Other Considerations: Hospitalized stage C/D HF patients are at increased risk for thromboembolic complications and should receive prophylactic anticoagulation with intravenous unfractionated heparin (Heparin sodium: 300-500 IU/kg i.v. (loading dose), then 100-300 IU/kg s.c. q 6-8 h) or low- molecular-weight heparin (Dalteparin: 100-150 IU/kg s.c. q8h) unless contraindicated. Warfarin is a reasonable in HF patients with antecedent embolic events or with paroxysmal or persistent atrial fibrillation [52].

Surgical Management of Refractory End-Stage HF (Stage D): Ventricular assist devices (VADs), cardiac transplantation, total artificial hearts, myocardial vascularization with coronary artery bypass grafting (CABG) and mitral valve repair or replacement are beneficial in stage D patients with demonstrable myocardial viability, in human [4,51].

Dogs with severe mitral regurgitation have poor prognosis even with all the pharmacological options that currently exist [53]. Recent improvements in cardiopulmonary bypass techniques have enabled its use in small breed dogs. In the case of prosthetic valves, matching the size of the valve and conquering thrombosis is essential for long-term prognosis. Recently, successful techniques for mitral valve repair with annuloplasty and chordal replacement have been utilized, which enables long-term survival for severe myxomatous mitral valve disease patients. Costs and availability are the major drawbacks of this option [54-57].

CONCLUSION

The aging process results in a host of physiological and

biological changes that lead to progressive HF. Nearly any CVD can lead to HF. HF in geriatric dogs is considered to be the result of structural and functional cardiac disorders that impair ventricular filling and ejection, and is broadly classified into: Diastolic heart failure is defined as HF with preserved ejection fraction (HF/PEF), systolic heart failure is also known as HF with low ejection fraction (HF/low-EF). Importantly, HF/PEF accounts for approximately 50% of all HF patients and its prevalence is higher in the elderly.

In humans, mortality and morbidity benefits for HF/low-EF have been reported with angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs), β-blockers and aldosterone antagonists, and the hydralazine-nitrate combination in patients intolerant to ACE inhibitors or nitrates. Therapies for symptom control and morbidity benefit include diuretics and digoxin. The most common and suggested therapy for dogs with HF by ACVIM panelists is furosemide, ACE-I, pimobendan. Pharmacological therapy is limited as the results of small trials have been inconclusive. To date, clinical trials in HF/PEF patients have not shown mortality benefit so that management is focused on symptom relief and treatment of the underlying cause.

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